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Title of Thesis: Characteristics of (^{3}H)2-Deoxyglucose Uptake by Slices of Rat Cerebral Cortex

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ABSTRACT

Title of Dissertation: Characteristics of [^{3}H]2-Deoxyglucose Uptake by Slices of Rat Cerebral Cortex

Jayne Kyle-Lillegard, Doctor of Philosophy, 1983

Dissertation directed by: Barry I. Gold, Ph. D., Assistant Professor, Department of Pharmacology

Glucose uptake across the brain cell membrane was modelled by the uptake of [^{3}H]2-deoxyglucose by rat brain slices. Net [^{3}H]2-deoxyglucose uptake was defined as the difference between the uptake of [^{3}H]-2-deoxyglucose (total uptake) and the uptake of L-[^{3}H]glucose (diffusional component) in parallel samples. L-[^{3}H]Glucose uptake by slices was a valid approximation of the space available for glucose diffusion because L-[^{3}H]glucose is an inactive stereoisomer of glucose and it distributed to a space essentially equal to total slice water.

[^{3}H]2-Deoxyglucose uptake was stereospecific, and net uptake was half-maximal at 1.85 mM. Net uptake was inhibited by other hexoses and showed a dependence on incubation temperature. It was not inhibited by phlorizin or by phloretin, two compounds known to inhibit glucose transport by kidney and by erythrocytes, respectively. Net [^{3}H]2-deoxyglucose uptake was not affected when various ions were individually deleted from the incubation medium. Uptake was inhibited by the oxidative phosphorylation uncoupler 2,4-dinitrophenol which suggested some dependence on mitochondrial energy. The correlation of uptake with adenosine 5'-triphosphate levels in the slices was not, however, straightforward. [^{3}H]2-Deoxyglucose uptake was increased as much as

three-fold after slices were preincubated for up to 60 min in the absence of exogenous D-glucose. It was also increased after slices were preincubated in the presence of pyruvate, or 3-O-methylglucose, but was not increased after slices were preincubated with 2-deoxyglucose. These results suggested that uptake was affected in some way at the hexokinase step. The relative amount of phosphorylated hexose accumulated by the slices was also increased after extended preincubation in the absence of exogenous D-glucose.

While these results are consistent with a carrier-mediated process, the phosphorylation reaction was not clearly differentiated from the transport process in these studies. Some of the results obtained may be explained by an effect on one or both of these processes.

A different approach to characterize the transport process was developed in a series of preliminary experiments which focused on the initial step of transport, that is, hexose binding to a carrier molecule. For these studies, a crude membrane fraction enriched in synaptic membranes was prepared from rat brain. D-[^{3}H]Glucose bound to this membrane fraction in a reversible, stereospecific manner. Binding was decreased in the presence of other hexoses known to be transported by the glucose carrier. D-[^{3}H]Glucose binding was also decreased when membranes were incubated with adenosine 5'-triphosphate or with glucose-6-phosphate. Results from these binding studies suggested at least two roles for the binding site: recognition of glucose by a carrier, or binding to hexokinase. Determination of this role awaits further study.

CHARACTERISTICS OF [^{3}H]2-DEOXYGLUCOSE UPTAKE

BY SLICES OF RAT CEREBRAL CORTEX

by

Jayne Kyle-Lillegard

Dissertation submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of the
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DEDICATION

To Mom and Dad, who have always been supportive and loving, and who are and will always be, my teachers. To my husband Wade, for his love and his patience, and his support at the times I needed him the most. To Dr. Barry I. Gold, advisor, teacher, and friend, for teaching me not only how to set up an experiment, but how to think.

To my family and my friends, who have made so many things easier, by helping me to take things a little less seriously. To the members of my dissertation committee, for their helpful suggestions and discussions. And to Steve Johnson, for his friendship and technical assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

ADP	adenosine 5'- diphosphate
ATP	adenosine 5'- triphosphate
B	bound
B_{max}	maximum number of binding sites
BSA	bovine serum albumin
Ci	curie
concn	concentration
CPM	counts per minute
DNP	2,4-dinitrophenol
DPM	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'tetraacetic acid
$F_{df,df}$	F ratio in analysis of variance
F	free
g	relative centrifugal force
g	gram
G-6-P	glucose-6-phosphate
h	hour
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid
K_D	equilibrium dissociation constant
K_i	inhibitor constant
K_m	Michaelis-Menten constant

k_{+1}	association rate constant
k_{-1}	dissociation rate constant
kcal	kilocalorie
KCN	potassium cyanide
$\uparrow K^+$KRB	high potassium Krebs-Ringer bicarbonate (modified)
KRB	Krebs-Ringer bicarbonate
KRP	Krebs-Ringer phosphate
l	liter
μ	micro, micron
m	milli
M	molar
min	minute
N	normal
OD_{595}	optical density at 595 nanometers
P	probability
P_2	crude mitochondrial pellet
P_2B	pellet described by Gray and Whittaker (1962), enriched in synaptosomes
"P_2B"	fraction enriched in synaptosomal membranes
P_2C	pellet described by Gray and Whittaker (1962), enriched in mitochondria
"P_2C"	fraction enriched in mitochondrial membranes
PSYN	purified fraction enriched in synaptosomal membranes
PSYN·MIT	purified fraction, resulting from preparation of PSYN, containing mitochondrial membranes
Q_{10}	temperature coefficient
s	second
SEM	standard error of the mean
V_{max}	maximal velocity

wt	weight
w/v	weight/volume
2DG	2-deoxyglucose
3-O-MG	3-O-methylglucose
°	degrees, Celsius

INTRODUCTION

One fact emerging from many studies of cerebral metabolism is that the brain is almost entirely dependent on glucose as an energy substrate (Quastel, 1969; Bachelard, 1970; Balázs, 1970; Maker et al., 1976). As blood passes through the brain, oxygen and glucose are extracted, and carbon dioxide and lactate are released (Gibbs et al., 1942). The respiratory quotient, the ratio of oxygen consumed to carbon dioxide produced, is close to 1.0 in brain, indicating that carbohydrates are the main source of fuel (Gibbs et al., 1942). Although the adult human brain accounts for only 3% of the total body weight, it accounts for approximately 20% of the total body utilization of glucose and oxygen (Bachelard, 1976). At rest, the adult human brain consumes glucose at a rate of 0.3 $\mu moles \cdot g^{-1} \cdot min^{-1}$, but during periods of increased electrical activity *in vivo* or *in vitro*, utilization of glucose by brain is greatly increased (Bachelard, 1976).

Evidence for the brain's dependence on glucose under normal circumstances comes from studies of the effects of hypoglycemia on brain function. Mann and Magath (1922) reported that hypoglycemia in animals caused coma, and that consciousness returned immediately after glucose was resupplied. Studies with rats showed that decreases in blood glucose below 2.2 $\mu mole \cdot g^{-1}$ resulted in electroencephalographic (EEG) changes, and blood glucose concentrations below 1.0 $\mu mole \cdot g^{-1}$ resulted in an isoelectric EEG and coma (Lewis et al., 1974a). In addition to acute changes in electrical activity, hypoglycemia caused pathologic changes in brain

tissue. Certain brain regions, such as cerebral cortex, are more sensitive to this effect than other brain regions (Wilkinson and Prockop, 1976). Hypoglycemia was associated with reduced cerebral glucose utilization, and severe hypoglycemia, accompanied by an isoelectric EEG, was associated with pronounced decreases in phosphocreatine and adenosine 5'-triphosphate levels in brain tissue (Norberg and Siesjö, 1976). In man, cerebral symptoms appear when the blood glucose concentration decreases below 2 mM (Bachelard, 1980).

Glucose is metabolized in brain mainly via glycolysis and the citric acid cycle to carbon dioxide and water (Quastel, 1969). The pentose phosphate pathway, also called the hexose monophosphate shunt, accounts for only a small amount of carbohydrate metabolism in brain (Quastel, 1969; Maker et al., 1976). Studies of [^{14}C]glucose metabolism showed that the predicted evolution of $^{14}CO_2$ was somewhat delayed, because of the highly active transaminases characteristic of adult brain (Maker et al., 1976). A small percentage of glucose is also used for lipid synthesis (2%) and for protein synthesis (0.3%; Maker et al., 1976).

Although the glycolytic pathway in brain is identical to that in other tissues (Balázs, 1970), the regulation of glucose flux through the pathway may be different. For example, in a review of cerebral carbohydrate metabolism, Balázs (1970) compared the activities of glycolytic enzymes in brain to those in liver, and stated that hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase, were each capable of greater maximal activity in brain compared to maximum rates in liver. These comparisons of enzyme activity in brain and liver are appropriate because the brain is an energy consuming organ while the liver is an energy storing organ (Balázs, 1970).

Brain glycogen concentrations ranging from 40 to 160 mg/100 g have been determined in a variety of experimental animals (Bachelard and McIlwain, 1969). By contrast, in humans skeletal muscle glycogen is 1% of its wet weight (Lehninger, 1975). The glycogen content of human liver can be approximately 6% of the wet weight of this organ, depending on the nutritional state (Guyton, 1976), about 90 g for a liver weight of 1500 g. The metabolic pathways for glycogen in brain are similar to those in other tissues (Bachelard, 1970), although the rates of synthesis are somewhat lower in brain than in muscle or liver (Bachelard and McIlwain, 1969). The role of glycogen in cerebral metabolism is that of a local energy reserve, glycogen synthesis and breakdown accounts for some 2% of the glycolytic flux (Maker et al., 1976). Brain glycogen could support normal rates of glycolysis for only approximately 25 minutes (Maker et al., 1976), and could maintain the reduced rate of glycolysis seen in coma for about 90 minutes, at which time irreversible brain changes occur (Quastel, 1969). Unlike in liver and in kidney, there is no appreciable gluconeogenesis from pyruvate or from lactate in brain (Bachelard, 1976).

Most of the brain's metabolic energy is used to maintain the resting membrane potential by actively pumping sodium and potassium ions (Bachelard 1976). When the neuronal activity of whole brain in vivo is increased by electrical stimulation, metabolic changes occur including increased glycolysis and respiration (McIlwain, 1950). This occurs in vitro as well; when brain slices are stimulated electrically, glycolysis and respiration can increase up to 10 times their resting rates (Bachelard, 1970). These are examples of the close association between neuronal activity and energy consumption which is characteristic of brain. Sokoloff

and his colleagues (1977) have used this principle as the basis of their [^{14}C]2-deoxyglucose autoradiography technique. In this technique, the uptake and phosphorylation of a radiolabelled glucose analogue, [^{14}C]2-deoxyglucose, by a brain region was shown to be closely correlated with the relative neuronal activity in that region. This metabolic mapping technique is widely used in neuroscience as an indication of regional functional activity.

Because brain metabolic activity is closely related to neuronal activity, it is apparent that regulatory factors coordinate the two processes. These regulatory steps could then be activated or inhibited in some way appropriate to the changing demands of the tissue. Many studies have been done to identify the regulatory steps in glycolysis. For example, Lowry et al. (1964) determined the effect of ischemia on the levels of glycolytic intermediates in mouse brain, and reported evidence for the control of glycolysis at the hexokinase and phosphofructokinase steps. Rolleston and Newsholme (1967) studied glycolysis in guinea pig cerebral cortex slices under selected conditions and identified at least 3 control points in glycolysis: hexokinase, phosphofructokinase, and pyruvate kinase. These glycolytic enzymes may also be subject to availability of substrates, cofactors, and ions, and to end-product inhibition, or to inhibition by other intermediates (Maker et al., 1976).

Hexokinase itself is a control point in glycolysis (Lowry et al., 1964; Rolleston and Newsholme, 1967). When normal rates for glycolysis *in vivo* (0.33 to 0.5 $\mu moles \cdot min^{-1} \cdot g^{-1}$ tissue) are compared with maximally activated rates of glycolysis *in vitro* (10 to 20 $\mu moles \cdot min^{-1} \cdot g^{-1}$), it is evident that under normal conditions the enzyme is operating at less than 5% maximal capacity (Lowry and Passonneau, 1964; Bachelard and Gold-

farb, 1969). Many factors have a role in the regulation of hexokinase activity. These include Mg^{2+}, adenosine 5'-triphosphate, adenosine 5'-diphosphate, glucose-6-phosphate, and glucose (Lowry and Passonneau, 1964). The subcellular distribution of hexokinase may also play a regulatory role. Brain hexokinase occurs in both a soluble form and a particulate form (associated with mitochondria). Although earlier studies showed differences in the activities of these two forms (Thompson and Bachelard, 1977), recent studies using purified hexokinase from the two subcellular locations showed that these forms were identical with respect to pH sensitivity, ability to bind to mitochondria, and kinetic activity (Needels and Wilson, 1983). However, glycolytic intermediates are known to affect the subcellular distribution of hexokinase (Bachelard, 1967; Wilson, 1968). The possibility remains that the subcellular localization of this enzyme may be important in control of glycolysis (Wilson, 1968; Bachelard, 1976).

Because some investigators believe the intracellular glucose concentration is very low (Bachelard, 1967; Bachelard and McIlwain, 1969; Bachelard, 1980), substrate availability may be a limiting factor for hexokinase activity (Lowry and Passonneau, 1964; Bachelard and McIlwain, 1969). Other researchers do not agree that intracellular brain glucose is very low. Siesjö (1978) calculated an intracellular concentration for free glucose of approximately 3 $\mu mole \cdot g^{-1}$ intracellular water. This calculation was based on an estimate of total brain glucose similar to that used by Bachelard (1967; 1971) to calculate intracellular glucose, but on somewhat different estimates of the contribution of extracellular space to whole brain volume, and different estimates of the concentration of glucose in the cerebrospinal fluid. Lund-Andersen (1980) estimated a

free intracellular glucose concentration of approximately 2 mM based on an equation for net flux of glucose from blood to brain. Some direct evidence for intracellular glucose in the spinal cord was reported by Passonneau and Lowry (1971) who measured free intracellular glucose in microdissected samples of large spinal neurons. The discrepancy regarding the concentration of intracellular glucose normally present in brain is a point of disagreement, and it relates to the question of the importance of glucose transport in the regulation of glucose metabolism. It is generally agreed (Siesjö, 1978; Lund-Andersen, 1979; Bachelard, 1980) that under conditions such as hypoglycemia or increased glycolysis resulting from seizure, ischemia, or hypoxia (Crane et al., 1981), glucose transport may become rate limiting for glucose utilization.

Glucose transport has also been shown to be affected by certain drugs, including amphetamines, barbiturates, cytochalasin B, and insulin. None of these drugs was shown to affect the phosphorylation reaction catalyzed by hexokinase when studied in vitro (see Bachelard, 1980). This suggests that glucose uptake, apart from phosphorylation, may influence glucose utilization under certain conditions (Bachelard, 1980).

Glucose transport by brain has been the subject of many investigations in the past 20 years. Fishman in 1964 and Crone in 1965 provided the first accounts that glucose transport to the brain was facilitated, displaying stereospecificity and saturability (Bachelard, 1975). Facilitated diffusion implies the existence of a carrier molecule for glucose; this carrier molecule could be located in the capillary endothelium, or in the membranes of neurons and glia (Lund-Andersen, 1979).

A variety of tissue preparations have been used to study glucose transport across neuronal membranes in vitro; these models include syn-

aptosomes, brain slices, and cell cultures. Synaptosomes are 'pinched-off' nerve endings resulting from homogenization and centrifugation of brain tissue in hypertonic sucrose (Gray and Whittaker, 1962); these endings are regarded as anucleate cells which contain all the necessary metabolic machinery (Bachelard, 1976). Brain slices are whole-cell-containing preparations which exhibit resting membrane potentials. Slices are advantageous to use because much of the original cytoarchitecture remains (Bachelard, 1976) and this tissue preparation does not rely on the vascular system for supply of nutrients (Bachelard, 1975). Disadvantages of this model include the large space available for diffusion (Lund-Andersen, 1979), which necessitates the use of appropriate controls, and the presence of glia. Cultured cells are usually transformed, and display vastly increased metabolic rates, so studies of cerebral metabolism in this tissue model are highly specialized (Lund-Andersen, 1979).

In vitro studies of glucose transport are complicated by the fact that glucose is readily metabolized. This problem is overcome by the use of glucose analogues; 2-deoxyglucose (2DG) and 3-O-methylglucose (3-O-MG) are frequently used. 2DG competes with D-glucose for transport into the cell, and for phosphorylation by hexokinase to form 2DG-6-phosphate. Unlike glucose-6-phosphate, however, 2DG-6-phosphate is not a substrate for phosphohexoseisomerase. Also, phosphatase activity in the brain is low. Therefore 2DG-6-phosphate is not metabolized to any extent. Sokoloff et al. (1977) showed that labelled 2DG-6-phosphate was not transported from brain to blood during a period of about 30 min following injection of [^{14}C]2DG, indicating that 2DG-6-phosphate remains trapped in the cell. Accumulation of this phosphorylated hexose can be measured by

liquid scintillation spectrometry or visualized by autoradiography, and used to approximate glucose uptake by the cell (Sokoloff et al., 1977). The analogue 3-O-MG also competes with glucose for transport into the cell (Bachelard, 1971), but it is not a substrate for hexokinase (Sols and Crane, 1954); 3-O-MG can leave the cell as the unmetabolized hexose.

In a review of glucose transport mechanisms in the kidney, Silverman (1976) briefly summarized some criteria by which glucose transport systems are classified. The two major classifications of glucose transport are represented by the erythrocyte and by the kidney or the intestine. Glucose transport by the erythrocyte is carrier-mediated; glucose is not transported against a concentration gradient (LeFevre, 1961). Transport by erythrocytes is not dependent on sodium (Silverman, 1976). Transport is, however, sensitive to inhibition by phloretin and by cytochalasin B (Silverman, 1976). Both these compounds have been shown to bind with high affinity to glucose uptake sites (LeFevre and Marshall, 1959; Lin and Spudich, 1974). Glucose transport by intestine or kidney is active; glucose is transported against a concentration gradient (Silverman, 1976). Glucose transport by these tissues occurs by cotransport with sodium (Crane, 1965; Silverman, 1976). Transport is sensitive to inhibition by phlorizin. High affinity binding of phlorizin was found to be associated with inhibition of galactose uptake by hamster intestine _in vitro_ (Stirling, 1967). Because these characteristics are clearly defined for the two major hexose uptake systems, the responses of other glucose uptake systems to the actions of phlorizin, phloretin, sodium, and cytochalasin B help to classify these systems. This approach has been used in many of the studies of brain hexose transport presented below.

Early _in vitro_ investigations of the mechanisms of glucose trans-

port by brain date back to 1965. Gilbert studied the uptake of xylose, a pentose, into the non-raffinose (intracellular) space of guinea pig brain slices (Gilbert, 1965; Gilbert, 1966; Gilbert et al., 1966). Joanny, Corriol, and Hillman (1969) reported saturable uptake of a number of sugars, including D-glucose, 2-deoxyglucose, [^{14}C]xylose, and arabinose by slices of guinea pig cerebral cortex. In these studies, with the exception of [^{14}C]xylose uptake, the amount of sugar accumulated by the tissue was measured enzymatically or by the reducing properties of the sugar. High concentrations of sugar were used to study kinetics and the K_m's were not interpretable from a physiological point of view.

Cooke and Robinson (1971) studied the uptake of [^{14}C]3-O-MG by rat cerebral cortex slices. They used lower concentrations of hexose than those used in the earlier studies reported above, but still reported a K_m = 205 mM. To explain this high constant, the authors discussed their results in terms of a model for 3-O-MG uptake which contained a diffusional component. Fishman and coworkers (1971) reported a similar K_m for 3-O-MG uptake by rat cerebral cortex slices.

Bachelard (1971) used a kinetic approach to estimate 2DG uptake by guinea pig brain slices. The uptake of radiolabelled 2DG was measured in the presence of different concentrations of D-glucose (5 to 15 mM), and results were expressed as μmoles sugar taken up per gram tissue. Values for uptakes were corrected for extracellular space estimated by raffinose uptake. Bachelard showed that D-glucose inhibited 2DG uptake and 3-O-MG uptake, but not the uptake of α-methylglucoside, a glucose analogue substituted at carbon 1. The K_m for 2DG uptake by guinea pig slices was 10 mM, and the K_i for glucose was 5 mM. These constants were the closest to physiological range (blood glucose concentration = 5 to 7

mM) that had been reported using the brain slice model in vitro. From the results of these competition studies, Bachelard suggested the importance of unsubstituted carbons 1 and 4 for affinity with the glucose carrier.

In the early 1970's, Diamond and Fishman (1973) in a lengthy report used synaptosomes to model glucose transport by brain cells, and reported a relatively high affinity constant for saturable 2DG uptake: K_m = 0.24 mM. This constant was much lower that any of the constants reported for hexose uptake by brain slices. Phloretin (K_i = 0.75 µM) was a competitive inhibitor of 2DG uptake by synaptosomes, and insulin was without effect. 3-O-MG inhibited 2DG transport with a K_i = 7 mM, but was not itself transported in detectable amounts by synaptosomes, which was in contrast to the measurable 3-O-MG uptake by slices reported by Cooke and Robinson (1971) and Bachelard (1971). A series of selective and non-selective metabolic inhibitors were shown to inhibit 2DG uptake by synaptosomes, these compounds included phenobarbital, KCN, DNP, iodoacetate, p-chloromercuribenzoate, and n-ethylmaleimide. Transport of 2DG was slightly inhibited in the presence of Na^+ or K^+ and was slightly increased in the presence of Li^+ (all 75 mM, Cl^- salts). $CaCl_2$ or $MgCl_2$ (both 1 mM) were without effect on uptake. The authors concluded from these results that none of the ions tested significantly affected 2DG uptake. A low affinity uptake system for 2DG was also detected in synaptosomes; the concentration of 2DG for half-maximal saturation was equal to 75 mM. Glucose inhibited this low affinity system with a K_i = 17.5 mM. The amount of free 2DG and 2DG-6-phosphate accumulated by synaptosomes was determined. In the high affinity uptake system, seventy-five percent of the accumulated hexose was in the phosphorylated form. This ratio was constant for a 5000-fold range of 2DG concentrations in

the medium (1 μM to 5 mM). These results were interpreted by the authors as evidence for uptake as a rate-limiting step for glucose utilization under these experimental conditions. In the low affinity system, the percentage accumulated as free 2DG did increase as a function of increasing concentrations of 2DG in the medium. Therefore, this low affinity system could be a measure of phosphorylation. Diamond and Fishman (1973) summarized their results by stating that synaptosomal uptake of 2DG was a sodium-independent, high affinity, carrier-mediated process. They postulated that such a process might serve to protect the high metabolic rate at the synapse from changes in blood glucose levels.

Heaton and Bachelard (1973) studied radiolabelled 2DG uptake by synaptosomes prepared from guinea pig cerebral cortex. They used the same kinetic approach used by Bachelard (1971) to study hexose uptake by brain slices. They found that low concentrations (0.35 mM) of D-glucose competitively inhibited 2DG uptake by synaptosomes; but that inhibition by D-glucose at higher concentrations (0.5 to 1.5 mM) was more complex. The authors suggested the possibility of a multivalent carrier system for hexose to explain these results.

Cultured human glioma cells (138 MG) and mouse neuroblastoma cells (C1300) were used to study hexose uptake (Edström et al., 1975; Walum and Edström, 1976a and b). Kinetic constants for hexose uptake by cells in the growth-inhibited state were K_m = 5.2 mM for [^{3}H]2DG uptake by glioma cells and K_m = 4.9 mM for [^{3}H]2DG uptake by neuroblastoma cells. Various compounds, including cytochalasin B and phloretin, affected the uptake of [^{3}H]2DG by neuroblastoma cells. Phlorizin, at a high concentration, also inhibited [^{3}H]2DG uptake by neuroblastoma cells. The uptake of [^{3}H]-3-O-MG by glioma cells in rapid growth phase was also inhibited by these

compounds. Preincubation of cells in the presence of iodoacetate and sodium cyanide (inhibitors of glycolysis and oxidative phosphorylation, respectively) caused inhibition of [^{3}H]2DG uptake but not of [^{3}H]3-O-MG uptake by neuroblastoma cells. The authors suggested that this effect was due to an inhibition of 2DG phosphorylation. In summary, evidence for a specific hexose uptake system was demonstrated in glioma and neuroblastoma cells. The reported K_m's for these systems were similar to the K_m for 2DG uptake by brain slices reported by Bachelard (1971).

Lund-Andersen and Kjeldsen (1976) studied the uptake of α-methylglucoside by slices of cerebral cortex. Others have studied the uptake of this analogue by brain slices and synaptosomes (Bachelard, 1971; Warfield and Segal, 1976). Lund-Andersen and Kjeldsen developed a three compartment model to study hexose uptake by brain slices. In this model, the incubation medium, the extracellular space, and the intracellular space represented the three compartments. The model was fitted to experimental data of hexose accumulation by slices and rate constants were determined for extracellular diffusion and for membrane transport. From these studies they showed that for α-methylglucoside the rate of extracellular diffusion was similar to that of membrane transport. For 3-O-MG, however, they showed that diffusion was rate-limiting. They concluded that α-methyl glucoside transport could be studied using cortical slices, but that of 3-O-MG could not. The authors suggested that the kinetic constants reported previously for sugar transport by brain slices were based on extracellular diffusion, and not on cell membrane transport.

In another study, Lund-Andersen and Kjeldsen (1977) used a four-compartment model to study and compare the uptake and the phosphorylation of 2DG. Their results indicated that diffusion across the extra-

cellular space was rate-limiting for transport, and that the transport rate exceeded the phosphorylation rate by a factor of ten. They suggested that saturation kinetics observed by others for 2DG were a measure of phosphorylation, and not of membrane transport.

Tan, Peterson, and Raghupathy (1977) used synaptosomes prepared from rat brain to study the uptake of D-[^{3}H]glucosamine. They demonstrated saturable uptake of this analogue with a K_m = 2.5 mM. In another study (Tan et al., 1978), D-glucosamine was shown to be rapidly phosphorylated after it was taken up by synaptosomes. Cytochalasin B inhibited uptake and phosphorylation to the same degree, and from these results the authors suggested that transport, and not subsequent phosphorylation, was rate-limiting under these conditions. The authors were unsure about the importance of a saturable uptake system for glucosamine in synaptosomes.

Renkawek et al. (1978) studied [^{3}H]3-O-MG uptake and [^{3}H]2DG uptake by organ cultures of cerebellum. The cultures contained a mixture of cell types including glia, neurons, and granular cells. Uptake of [^{3}H]3-O-MG was linear for 60 min when corrected for nonspecific diffusion measured by simultaneous uptake of radiolabelled sucrose or inulin. Uptake kinetics were studied at 30 min, when the percent nonspecific diffusion was at a minimum value. A K_m = 11 μM and K_m = 4 μM were determined for the uptake of [^{3}H]3-O-MG and [^{3}H]2DG, respectively. These values are about one-hundred times lower than values reported for 2DG uptake by synaptosomes (Diamond and Fishman, 1973; Heaton and Bachelard, 1973). Uptake of [^{3}H]3-O-MG by cerebellar cultures was inhibited by 5 mM D-glucose, 5 mM 2DG, or 5 mM xylose. These concentrations are quite high when compared to the concentrations of radiolabelled hexose used. Phlorizin, at 1 mM was also inhibitory, but deletion of sodium from

the incubation medium was without effect. The authors concluded that their results were indicative of a high affinity uptake system for these hexose analogues in organ cultures of cerebellum, and they suggested that the low K_m values might have been a result of their experimental technique.

Fletcher and Bachelard (1978), using low [^{14}C]2DG concentrations (0.5 to 1.5 mM), described a high affinity uptake system for radiolabelled 2DG by slices of guinea pig cerebral cortex. They used the kinetic approach described for 2DG uptake by slices (Bachelard, 1971; Heaton and Bachelard, 1973). For these studies, low concentrations (0.1 to 1.5 mM) of glucose were present during the uptake period. Because they were concerned about the effects of low glucose on slices, they added sodium pyruvate as an oxidizable substrate in some studies and obtained similar results. Kinetic data obtained from these systems resembled data from 2DG transport by synaptosomes (Heaton and Bachelard, 1973). Double reciprocal plots of [^{14}C]2DG uptake were linear in the presence of 0.1 to 0.5 mM D-glucose, but were curvilinear in the presence of higher glucose concentrations. Insulin affected the shape of these competition curves. It had no effect on the low affinity system also present in brain slices. From these studies, the investigators suggested that the high affinity uptake of 2DG demonstrated in synaptosomes was not an artifact of the preparation. Also, the glial membrane may be the site for low affinity hexose transport, and the capillary endothelium and the neuronal membrane may be the site for the high affinity transport system.

Synaptosomes prepared from cerebral cortex of Long-Evans rats transported radiolabelled 2DG similarly to synaptosomes prepared from Sprague-Dawley rats, according to a study done by Wheeler and Hollings-

worth (1979). These researchers showed that hexose uptake by synaptosomes was dependent on mitochondrial energy; their results are similar to those reported by Diamond and Fishman (1973). Data from another series of experiments showed that sodium ion was an inhibitor of 2DG transport by synaptosomes. Uptake data obtained in the presence of a range of sodium concentrations was best fitted to a model describing noncompetitive inhibition by sodium. While sodium effects on V_{max} and K_m for uptake were not profound, the authors suggested that they may be important at the synapse.

Halton, Taylor, and Lopes (1980) described a saturable rapid uptake of [^{3}H]3-deoxy-3-fluoroglucose ([^{3}H]3DFG) by rat brain synaptosomes. The K_m = 0.62 mM estimated for [^{3}H]3DFG uptake was similar to that for [^{3}H]2DG uptake reported by others (Diamond and Fishman, 1973; Wheeler and Hollingsworth, 1979). D-glucose, cytochalasin B, and phloretin inhibited [^{3}H]3DFG uptake, but ouabain and phlorizin were without effect. Addition of Na^+, K^+, Mg^{2+} or Ca^{2+} to the incubation medium was also without effect on uptake. Because 3DFG is not phosphorylated to a great extent, it was of interest to compare the effects of certain metabolic inhibitors on the uptake of this analogue with results obtained by others using 2-deoxyglucose. Under experimental conditions favoring maximal phosphorylation of [^{3}H]3DFG, a variety of metabolic inhibitors including KCN, antimycin A, DNP, and rotenone caused inhibition of [^{3}H]3DFG uptake and subsequent phosphorylation. In another set of studies, ATP was added to the incubation mixture in the presence of 10 mM KCl (to aid entry of ATP). With ATP, a reversal of the inhibition caused by the metabolic inhibitors of both uptake and phosphorylation was seen. All these results were summarized to suggest that the transport of [^{3}H]3DFG was not energy dependent, because changes in uptake caused by metabolic inhibitors were always

associated with changes in phosphorylation. Furthermore, after the initial rate of uptake, phosphorylation became rate-limiting for uptake.

Keller, Lange, and Noske (1981) studied glucose uptake in cultured glioma- (C6) and neuroblastoma- (C1300, N2A) derived cells. They incubated cells in the presence of 5 to 15 mM D-glucose for 3 h and measured (spectrophotometrically) the difference between D-glucose concentrations in the medium at the beginning and end of the incubation. Intracellular D-glucose was also measured under these steady state conditions. The authors found that glioma cells had a lower level of intracellular glucose, under steady state conditions, when compared to neuroblastoma cells. The lower level in glioma cells was due to a lower rate of glucose uptake as measured by the change of glucose concentration in the medium after 3 h of incubation. Initial flux of glucose by C1300 and C6 cells was also measured. Influx was determined by incubating cells for 3 h in the absence of glucose. Iodoacetate was added to inhibit glycolysis. After this period, cells were rinsed and 10 mM glucose was added. The accumulation of glucose and phosphorylated intermediates was measured at 20 and 40 s. The rate of glucose influx was greater in neuroblastoma than in glioma cells. The authors stated that, under the experimental conditions used, influx of glucose was more rapid than phosphorylation in neuroblastoma.

Although many characteristics of the brain glucose transport system have been revealed in part by the _in vitro_ studies outlined above, very little is known about the mechanisms involved in the regulation of this system. Glucose transport in most mammalian organs proceeds by two carrier-mediated mechanisms: facilitated diffusion and active transport. For example, epithelial cells in the intestinal mucosa and in the proximal

tubule of the kidney both transport glucose actively at the expense of metabolic energy. In a review of glucose transport mechanisms, Elbrink and Bihler (1975) described two types of facilitated diffusion: regulated and non-regulated. The major bases for this classification were intracellular glucose concentration and variability of metabolic activity. An example of a regulated glucose transport system is glucose transport by muscle. In muscle, there is little free glucose, and metabolism varies according to contractile activity. Insulin stimulates sugar transport in muscle, and glycogen is present as a reserve fuel. The erythrocyte transport system for glucose is an example of a non-regulated system; there is free glucose intracellularly, and metabolism, to maintain cell structure, is steady. Erythrocytic glucose transport does not respond to insulin (Elbrink and Bihler, 1975).

Brain cells transport glucose in a carrier-mediated, stereospecific manner. There is some evidence suggesting that this transport is dependent on a source of metabolic energy (Diamond and Fishman, 1973; Wheeler and Hollingsworth, 1979). If transport is dependent on energy then the transport system would resemble the active transport system in the intestine or in the kidney. The question of whether glucose transport or phosphorylation by hexokinase is rate-limiting for glucose utilization by brain cells has not been answered. If transport is rate-limiting for utilization, then substances or events which alter glucose transport would directly affect neuronal function. Finally, an understanding of some of the factors which regulate the transport of glucose by brain cells might lend insight to the mechanism of action of some drugs which interfere with brain glucose transport or with cerebral glucose utilization.

Specific Aims

Glucose transport by brain cells is a carrier-mediated process. While many characteristics of this process have been revealed by *in vitro* studies, the regulation of this system has not been well defined. The aim of this research has been to learn more about the characteristics and regulation of brain glucose transport using an *in vitro* brain slice model. This model was based on the uptake of the glucose analogue [^{3}H]2-deoxyglucose ([^{3}H]2DG) by rat cerebral cortex slices under selected experimental conditions. Using this model, I determined the correlation between intracellular levels of ATP and [^{3}H]2DG uptake to reveal whether the uptake process was dependent on metabolic energy. I tested the effect on [^{3}H]2DG uptake of *in vitro* conditions which mimic physiological increases in neuronal firing rates to determine whether rate of uptake would increase as a function of neuronal activity. I studied the formation of [^{3}H]2DG-6-phosphate, the product of hexokinase catalysis, and compared it to the uptake of [^{3}H]2DG under different experimental conditions. To characterize [^{3}H]2DG uptake by slices and compare it to glucose uptake systems in other tissues, I determined the effect of selected pharmacological agents, such as phloretin, phlorizin, and DNP, which are known to affect glucose transport in other tissues, on [^{3}H]2DG uptake by brain slices.

Carrier-mediated transport involves the reversible binding of the solute to a carrier molecule which transports the solute across the cell membrane. To study this step of glucose transport by brain, a membrane model was used. This model was based on the binding of D-[^{3}H]glucose to crude membrane fractions prepared from rat cerebral cortex. Using this model, I determined whether glucose binding was stereospecific and inhib-

ited by glucose analogues which inhibited [^{3}H]2DG uptake by slices. I designed studies to determine the function of this binding site.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (140 to 320 g) were purchased from Charles River Laboratories (Wilmington, Massachusetts), housed four to a cage, and maintained on a 12/12 h light/dark cycle, with free access to food and water ad lib.

The following compounds were obtained from Sigma Chemical Company (St. Louis, Missouri): adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), D-glucose-6-phosphate (G-6-P), 2-deoxy-D-glucose (2-deoxyglucose, 2DG), 2-deoxy-D-glucose-6-phosphate, 2,4-dinitrophenol (DNP), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES), yeast hexokinase, L-glucose, 3-O-methyl-D-glucopyranose (3-O-methylglucose, 3-O-MG), phloretin, phlorizin, and sodium azide. D-glucose was purchased from Mallinckrodt, Inc. (St. Louis, Missouri). Radiochemicals, including 2-[1,2-^{3}H]deoxy-D-glucose, L-[1-^{3}H(N)]glucose, D-[2-^{3}H(N)]glucose, 3-O-[methyl-^{3}H]glucose, and D-[1-^{14}C]glucose-6-phosphate, disodium salt, and Protosol, Aquassure, and Aquasol were purchased from New England Nuclear (Boston, Massachusetts). Deoxyadenosine 5'-[α-^{32}P]-triphosphate, triethylammonium salt ([^{32}P]ATP) was purchased from Amersham (Arlington Heights, Illinois). Dye-reagent concentrate for protein determination and AG1-X8 resin were obtained from BioRad Laboratories (Richmond, California), and and Ready-Solv EP was obtained from Beckman Instruments (Fullerton, California). All other chemicals and reagents

were supplied by commercial sources.

Methods

Transport Studies

Slice preparation

A schematic representation of the method used to prepare brain slices is shown in Figure 1. Male rats were decapitated, and the brains were rapidly removed to a 0.9% saline-moistened filter paper placed on an ice-filled Petri dish. Stainless steel spatulas were used to peel away cerebral cortex from basal ganglia and underlying brain structures, and to cut the cortex into small pieces. Pieces of cortex were weighed on an electronic top-loading balance (Sartorius Model 1205 MP, Brinkmann Instruments Inc., Westbury, New York); wet tissue weights ranged from 30 mg to 55 mg. Each piece of cortex was then sliced with a Smith and Farquhar Tissue Sectioner (Model TC-2, Sorvall Inc., Newtown, Connecticut) set at 225 μ, and slices were transferred to glass vials containing 4.95 ml of a modified Krebs-Ringer-bicarbonate medium (KRB) which contained 124 mM NaCl; 5 mM KCl; 1.3 mM $MgSO_4$; 1.25 mM KH_2PO_4; 1.25 mM $CaCl_2$; and 26 mM $NaHCO_3$, and had been bubbled with 95% O_2/5% CO_2 before it was added to the vials. Each vial of KRB was warmed to the 25° incubation temperature before slices were added, and slices were dispersed by brief vortexing or by gentle aspiration through a modified pasteur pipette. This brain slice procedure lasted approximately 10 min.

Transport assay

A flow chart of the transport assay is shown in Figure 2. Slices were equilibrated at 25° in a Dubnoff metabolic shaking incubator (Precision Scientific Group, Chicago, Illinois), set at 52 strokes per min,

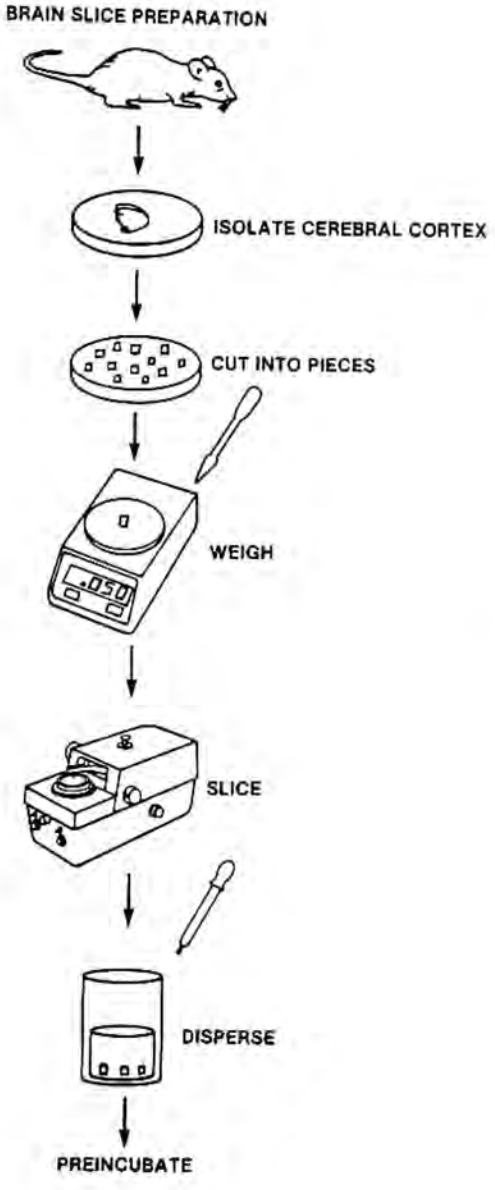

Fig. 1. Brain slice preparation

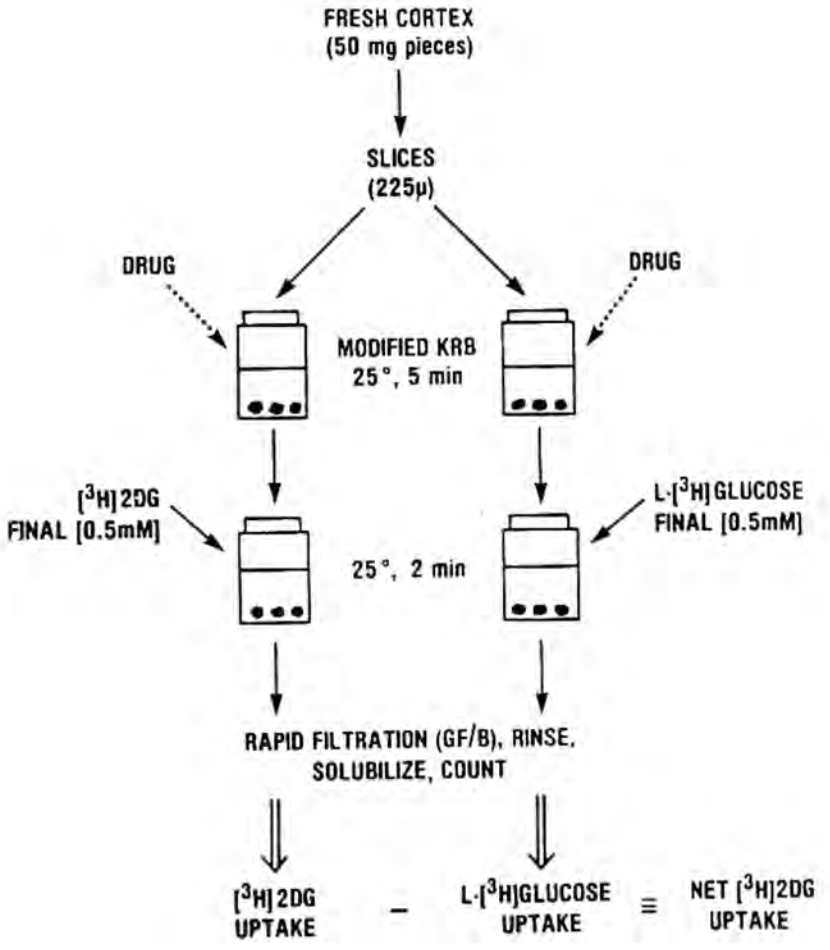

Fig. 2. Transport assay

for 5 min in the absence of substrate. Transport was initiated by adding 50 µl of either [^{3}H]2-deoxyglucose ([^{3}H]2DG) or L-[^{3}H]glucose (both 0.1 µCi/µmole) to a final concentration, unless otherwise noted, of 0.5 mM, and slices were incubated for 2 to 5 min at 37° in early experiments. The incubation temperature was changed to 25° in later experiments because of results obtained in a study of the effect of incubation temperature on net transport. Incubation was stopped by rapid filtration of slices over glass fiber filters (Whatman, GF/B), and tissue was rinsed with 8 ml 0.9% saline (25°). Filters containing slices were transferred to plastic scintillation vials, slices were solubilized with 1.5 to 2 ml Protosol, and 0.068 ml glacial acetic acid and 10 ml scintillation cocktail were added. Radioactivity was estimated by liquid scintillation spectrometry in a Packard Liquid Scintillation Spectrometer B2450.

Transport assay, preincubation conditions

The protocol followed for preincubation of slices and assay of subsequent hexose transport is outlined in Figure 3. Slices of cortex, prepared as described above, were preincubated at 25° in a Dubnoff metabolic shaking incubator under a gassing hood in a 95% O_2/5% CO_2 atmosphere (flow rate of 0.24 liters per min) for a time which varied with the experimental protocol. After preincubation, slices were filtered over nylon mesh as described by Gold et al. (1978), rinsed with 5 ml warm KRB containing either 10.4 mM L-glucose or 10.4 mM choline chloride, and transferred to clean vials containing fresh 25° KRB with L-glucose or choline chloride. Slices were equilibrated for 5 min, and the transport reaction was initiated as described in the previous section. In some experiments, slices were suspended in porous-bottomed plastic baskets lined with filters cut

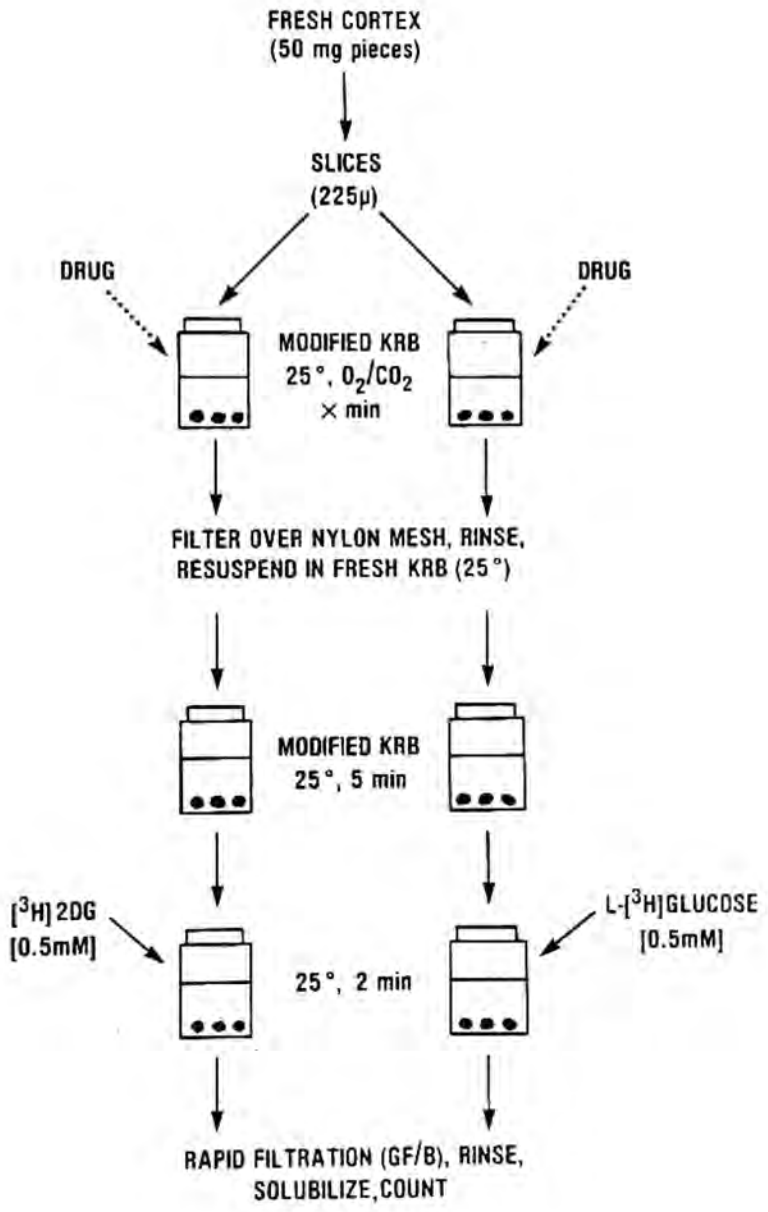

Fig. 3. Transport assay, preincubation conditions

from Kimwipes, and the baskets were set in glass incubation vials. These samples were transferred to clean vials after preincubation by removing the basket containing the slices to a vacuum apparatus to rapidly drain the preincubation medium, rinsing the slices with 2 to 3 ml of 25° KRB containing 10.4 mM choline chloride, and placing the basket containing the slices in a clean vial containing 25° incubation medium. Control values for [^{3}H]hexose transport were obtained from samples preincubated in the absence of drug, or from samples which were not preincubated. In later experiments, a 30 min preincubation period in the presence of 10.4 mM D-glucose was included for all samples, and a flowchart of this procedure is shown in Figure 4.

ATP measurement

The method used to measure endogenous levels of ATP was slightly modified from that described by Cooney et al. (1974). In this technique, radiolabelled glucose-6-phosphate formed from the phosphorylation by hexokinase of added D-[^{3}H]glucose is directly proportional to the amount of ATP present. After preincubation and equilibration, slices were drained rapidly and placed in vials containing 2 ml of 100 mM HCl in methanol to precipitate ATP. Denatured slices and the methanol solution were then transferred to polypropylene centrifuge tubes and disrupted with a Polytron (Brinkmann Instruments, Westbury, New York). Samples were centrifuged (Beckman J-21B or Sorvall RC-5B centrifuge) for 10 min at 17,000 g and the resulting pellets, containing the ATP, were resuspended in 2 ml methanol. This suspension was centrifuged at 35,000 g for 10 min, and the final pellets were resuspended in a 5 or 6 ml solution of 100 mM Na_2CO_3 containing 250 µM EDTA to extract ATP. Supernatants obtained after

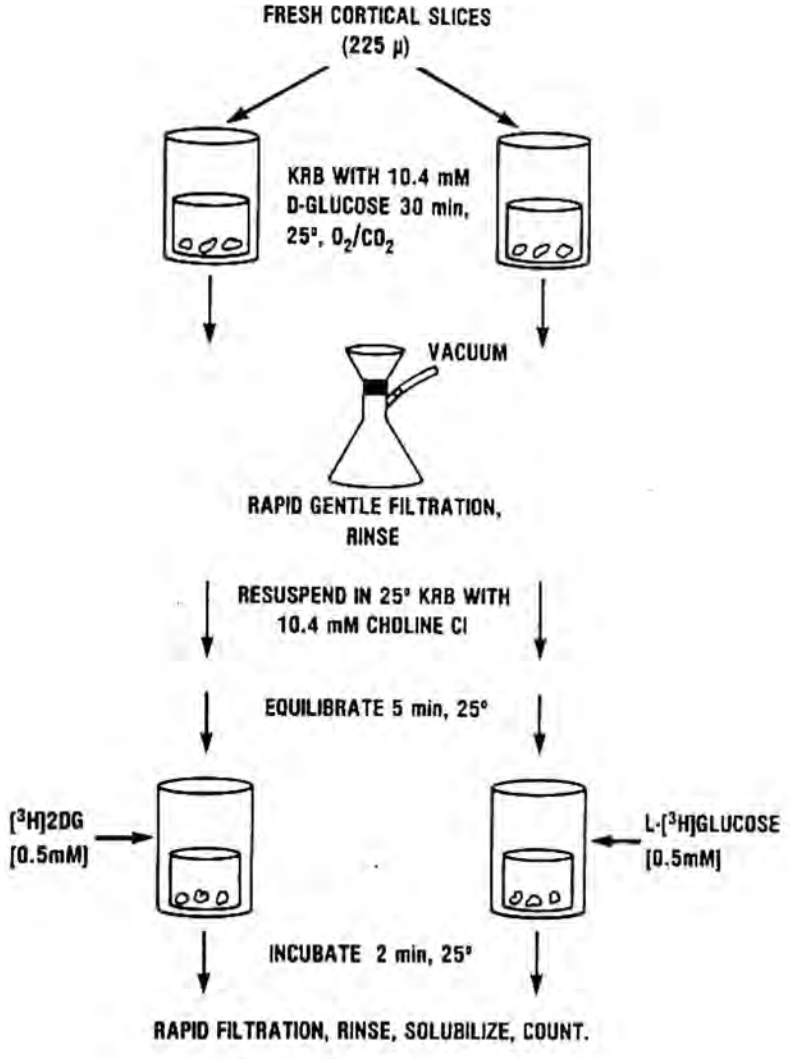

Fig. 4. Modified preincubation assay for hexose transport

centrifugation of this suspension for 10 min at 35,000 g were used for ATP measurement. ATP recovery during the extraction procedure was measured by adding a known amount of [^{32}P]ATP to tissue slices which had been homogenized in methanol containing HCl. A fraction of this suspension was removed for estimation of total radioactivity, the remainder was centrifuged, and the extraction procedure was continued as described above. A fraction of the final supernatant was removed for estimation of recovered radioactivity. The radioactivity present in the final supernatant, corrected for dilution, was divided by the total radioactivity in the original suspension. Recovery of ATP by this extraction procedure was 28%. Five microliter aliquots of supernatant were added to Eppendorf centrifuge tubes, and then 10 µl of Cooney's reagent mixture containing D-[^{3}H]glucose, $MgCl_2$, yeast hexokinase and Tris-HCl (pH 8.4 at 20°) were added. The samples were incubated for 1 h at 37°. After incubation, 5 mM barium acetate in 70% ethanol and a 1% solution of glucose-6-phosphate were added, and the tubes were centrifuged (Beckman Microfuge B) for 3 min to precipitate the glucose-6-phosphate. The supernatants were discarded, the pellet was washed with ethanol, and a 1 N solution of H_2SO_4 was added to solubilize the pellet. Samples were vortexed briefly and placed in a 95° heating block for 15 min. The tubes were vortexed a second time, the caps were removed, and the tubes were placed in plastic scintillation vials. Twelve ml of Ready-Solv EP scintillation cocktail were added, and radioactivity was estimated by liquid scintillation spectrometry. ATP values for the samples were estimated from an experimental standard curve determined with known concentrations of ATP; a typical standard curve is shown in Figure 5. Values are expressed as pmoles ATP/µg protein in the original homogenate, uncorrected for loss during extraction.

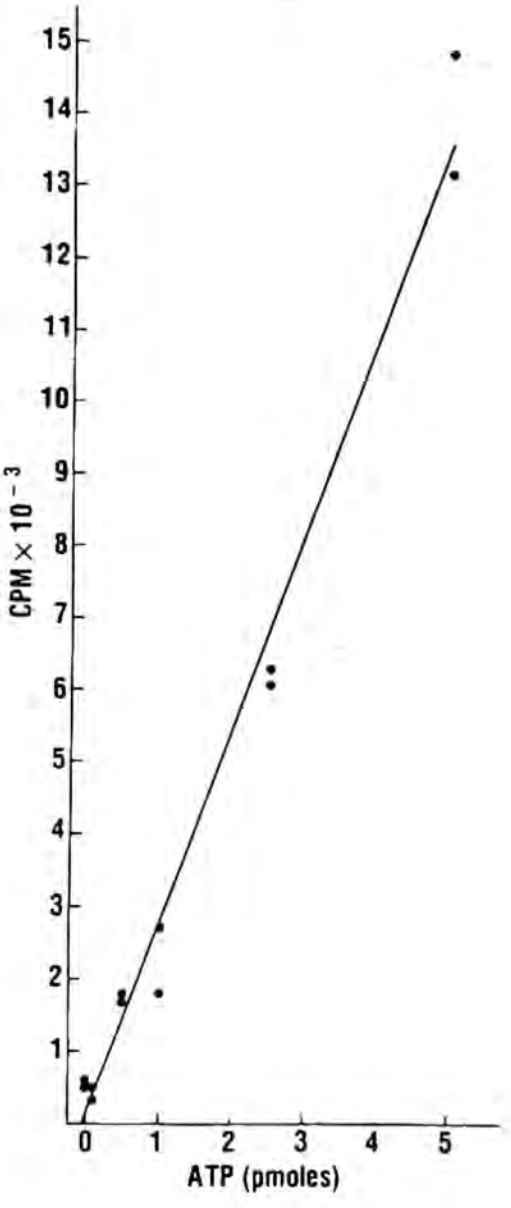

Fig. 5. Standard curve for ATP measurement. Each point represents a single sample. Line was estimated by linear regression.

Ion exchange chromatography for separation of neutral and phosphorylated hexoses

Brain slices were preincubated for 30 min in 25° KRB containing 10.4 mM D-glucose, rinsed, and transferred to clean vials containing 25° KRB with 10.4 mM choline chloride. Samples were incubated with 0.5 mM [^{3}H]2DG or with 0.5 mM L-[^{3}H]glucose (both 0.4 μCi/μmole) for time periods determined by the experimental protocol. After incubation, baskets containing the slices and filters were transferred to a vacuum apparatus, slices were rinsed with 8 ml 25° 0.9% saline, and the filters with adherent slices were transferred to Corex tubes containing 2.8 ml 0.4 N perchloric acid. Tissue slices from two replicate samples were pooled. Tissue was disrupted by sonication (Model W-220F, Heat Systems-Ultrasonics, Inc., Plainview, New York), and samples were centrifuged (Beckman J-21B) at 3,000 g for 15 min. A 1.2 ml fraction of the resulting supernatant was removed and titrated with KOH to pH 7.0 (pH paper end-point) to precipitate $KClO_4$. After neutralization, samples were centrifuged for 15 min at 3,000 g. One ml fractions of the resulting supernatants were applied to small columns of AG1-X8 resin (1 ml bed volume, Cl^- form, 200-400 mesh; BioRad, Richmond, California). An elution profile of authentic [^{3}H]glucose and [^{14}C]glucose-6-phosphate is shown in Figure 6. Neutral hexose was eluted with distilled water, and the first and second fractions were collected and pooled. Phosphorylated hexose was eluted with 0.5 N HCl, and two 1 ml fractions were collected and pooled. Ready-Solv EP scintillation cocktail, 14 ml, was added to each pooled fraction, and radioactivity was estimated by liquid scintillation spectrometry. Results were expressed as percent of the total [^{3}H] recovered.

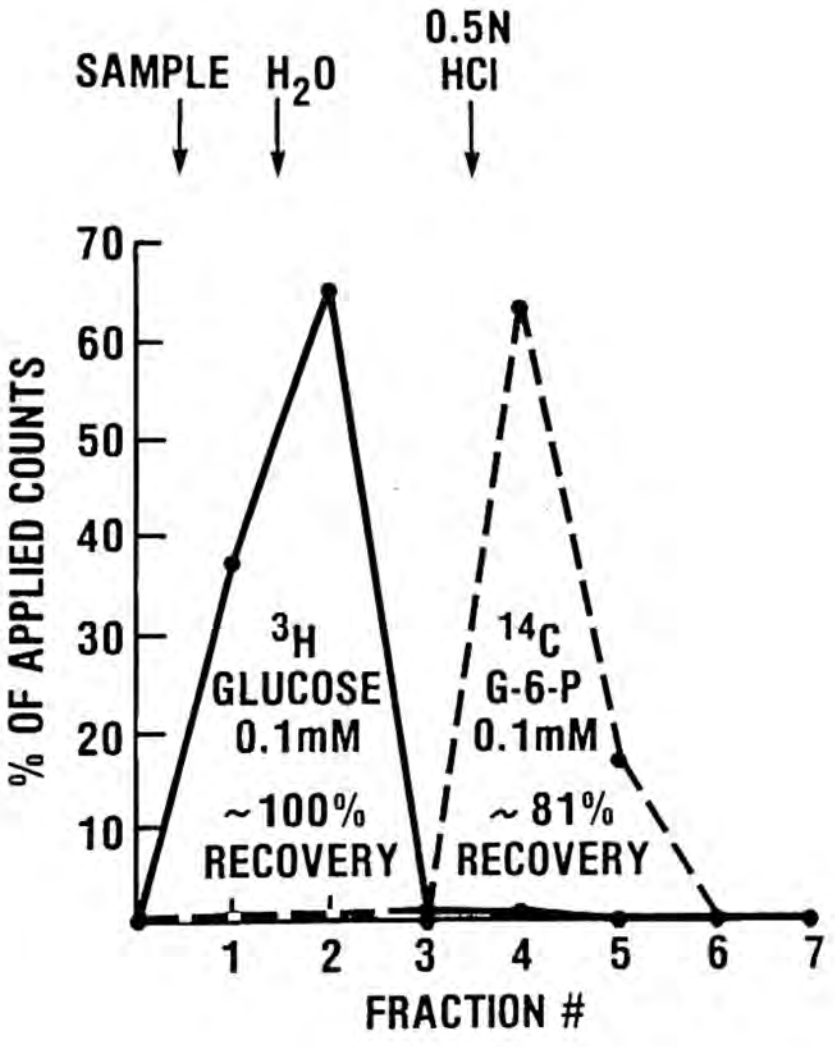

Fig. 6. Elution profiles of authentic [^{3}H]-glucose and [^{14}C]-glucose-6-phosphate

Binding Studies

Membrane preparation

A flow chart of this method is shown in the right-half of Figure 7. Cortices from several rats were dissected, pooled, and homogenized in ice-cold 0.32 M sucrose (5% w/v) with a Potter-Elvehjem Teflon-glass homogenizer (Kontes, Vineland, New Jersey) and a motor driven laboratory stirrer (Talboys Engineering Corporation, Emerson, New Jersey). Preparation of synaptic membrane-enriched fractions was according to the method of Enna and Snyder (1975). All centrifugations were done in polypropylene tubes in a Beckman J-21B or Sorvall RC-5B centrifuge at 4°. Brain homogenates were centrifuged at 1,000 g for 10 min to remove unbroken cells, nuclei, and erythrocytes. The supernatants were decanted into clean tubes and centrifuged for 20 min at 20,000 g. The resulting pellets, the crude mitochondrial fractions, were resuspended in ice-cold distilled water (5% w/v) with a Polytron and centrifuged at 8,000 g for 20 min. The resulting supernatants and buffy uppercoats were decanted into clean tubes, recentrifuged at 48,000 g for 20 min, and the pellets obtained were washed once by resuspension in distilled water and recentrifugation. The final pellets were used the same day or stored frozen at -70°.

Preparation of purified membranes

A flow chart of this membrane preparation is shown in the left-half of Figure 7 and in Figure 8. Synaptosomal fractions and mitochondrial fractions were purified by Gray and Whittaker's (1962) method; and all centrifugations were at 4°. Cerebral cortex was homogenized with a Potter-Elvehjem homogenizer driven by a motorized laboratory stirrer in

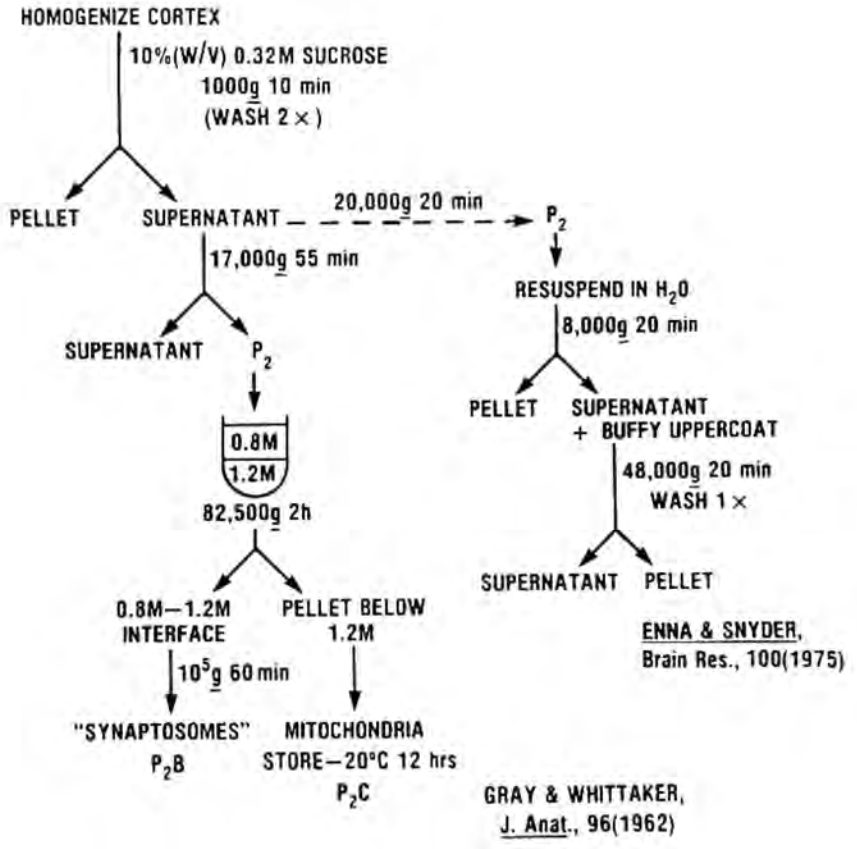

Fig. 7. Preparation of synaptic membranes

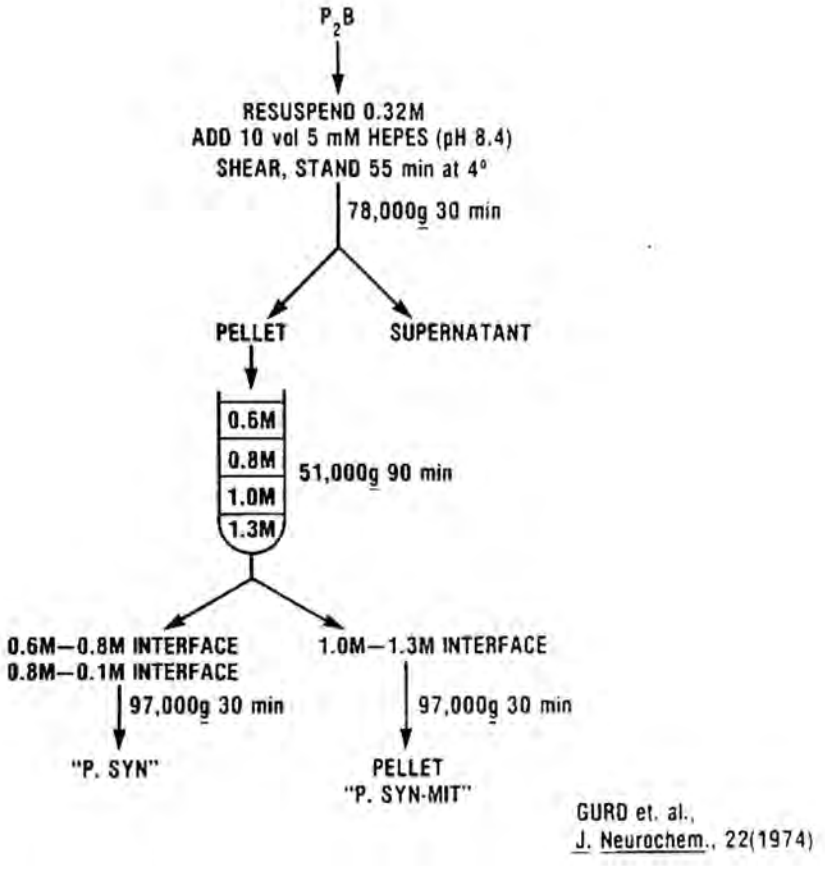

Fig. 8. Preparation of purified membranes

0.32 M sucrose (10% w/v), and these crude homogenates were centrifuged at 1,000 g for 10 min. The supernatants were decanted into clean tubes, and the pellets were washed twice by resuspension in 0.32 M sucrose and recentrifugation. The supernatants from the washes were pooled with the first supernatant and these were centrifuged at 17,000 g for 55 min. The resulting crude mitochondrial pellet, called P_2, was homogenized in 0.32 M sucrose (25% w/v) and layered on a two-step sucrose gradient (0.8 - 1.2 M). The gradients were centrifuged at 82,500 g for 2 h in a Beckman SW 27 rotor and L5-50 ultracentrifuge. The 0.32 M sucrose and 0.8 M sucrose bands were aspirated, and the material collecting at the interface of 0.8 M and 1.2 M sucrose was transferred to polycarbonate centrifuge tubes, diluted with ice-cold distilled water, and centrifuged at 100,000 g for 1 h in a 50.2 Ti rotor (Figure 7). The pellet resulting from this centrifugation, called P_2B, has been shown to be a relatively pure synaptosomal fraction (Gray and Whittaker, 1962). It was resuspended in a minimum volume of 0.32 M sucrose, and stored at -20° overnight. The pellet formed during centrifugation of the two-step sucrose gradient, called P_2C, has been shown by Gray and Whittaker (1962) to be a relatively pure mitochondrial fraction. This mitochondrial fraction was stored at -20° overnight, and then it was resuspended in 25 mM HEPES, pH 7.4 for binding studies ("P_2C"). Further purification of brain synaptosomal fractions was as described by Gurd et al. (1974), but the synaptosomal fraction used, P_2B, was prepared as described above, and not as described by Gurd. Brain synaptosomal fractions were prepared, resuspended in a minimum volume of 0.32 M sucrose, and frozen at -70°. The following morning, the fractions were thawed, and 20 to 30 ml of 5 mM HEPES buffer (pH 8.4 at 20°) was added added to each tube. The samples were sheared

(pulled through a 14 gauge cannula) 5 times, incubated for 55 min at 4°, and sheared again. After incubation, samples were centrifuged at 78,000 g for 30 min, and some of the resulting pellets were stored on ice. These pellets were homogenized in 25 mM HEPES, pH 7.4, and were used in the binding studies as the synaptic membrane-enriched fraction ("P_2B"). The remaining pellets were resuspended in a minimum volume of 0.32 M sucrose, layered over a four-step sucrose gradient (0.6 - 0.8 - 1.0 - 1.3 M), and centrifuged at 51,000 g for 90 min. The top layer was aspirated, and the material which collected at the 0.6 - 0.8 M interface, and at the 0.8 - 1.0 M interface was collected, pooled, diluted with distilled water, and centrifuged at 97,000 g for 30 min. The resulting pellet, when resuspended in 25 mM HEPES, pH 7.4, was used in subsequent binding studies as the purified synaptic membrane-enriched fraction (PSYN). Material which collected at the 1.0 - 1.3 M interface was diluted with distilled water at 4°, centrifuged at 97,000 g for 30 min, and resuspended in 25 mM HEPES, this fraction was the purified synaptosomal mitochondrial-containing fraction (PSYN·MIT) in binding studies.

D-[^{3}H]Glucose binding to membrane fractions, centrifugation assay

The protocol used to study D-[^{3}H]glucose binding is illustrated in Figure 9. Membrane pellets were resuspended with a Polytron in ice-cold 25 mM HEPES (pH 7.4 at 20°) and the suspension was kept on ice. Two ml fractions of membrane suspension, or 1 ml of membrane suspension and 1 ml HEPES buffer were transferred to plastic centrifuge tubes. To one set of tubes excess D-glucose was added, and an equivalent volume of HEPES buffer was added to a parallel set of tubes. All samples were warmed for 3 min at 37° in a shaking water bath, and the binding reactions were initiated

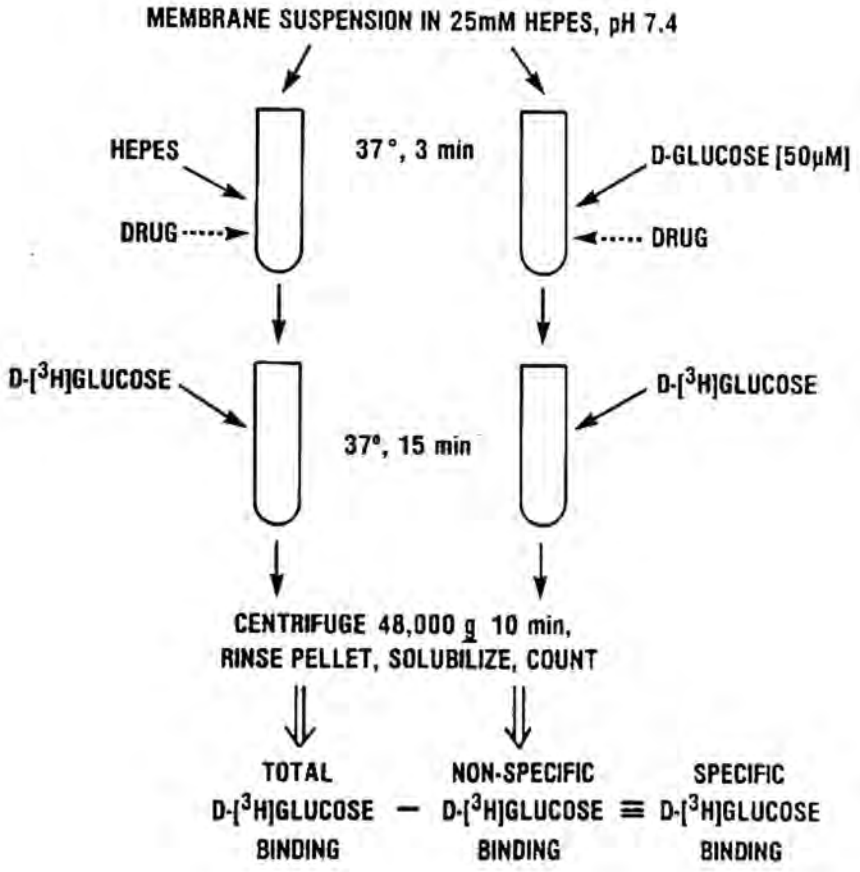

Fig. 9. D-[^{3}H]-Glucose binding to membrane fractions, centrifugation assay

by adding D-[^{3}H]glucose to a final concentration of 14.1 to 15.2 nM (17.3 to 16.0 Ci/mmole, respectively). Incubations were for 15 min at 37°, and the reactions were stopped by centrifugation (48,000 g for 10 min at 4°). The resulting pellets were rinsed twice, gently so as not to disturb the pellet, with 5 ml ice-cold distilled water, and the tubes were carefully swabbed with Kimwipes. Protosol (1.5 ml) was added to each tube to solubilize the pellet, glacial acetic acid (0.068 ml) and Ready-Solv EP (5 ml) were then added, and the mixture was transferred to a plastic scintillation vial. Each tube was rinsed with an additional 5 ml of scintillation cocktail, and this cocktail was added to the scintillation vial. Radioactivity was estimated by liquid scintillation spectrometry in a Packard Liquid Scintillation Spectrometer B2450, or a Beckman LS 9800 Liquid Scintillation Counter. Radioactivity was expressed in DPM, calculated by the external standard channels ratio method or by the Beckman data reduction package. Results were expressed as pmoles D-[^{3}H]-glucose bound per mg protein in the membrane suspension.

D-[^{3}H]Glucose binding to membrane fractions, filtration assay

In an effort to increase daily experimental capacity, a filtration assay for binding studies was developed. D-[^{3}H]glucose binding to crude membrane fractions prepared from rat brain was measured as described above, but the reaction was stopped by rapid filtration of the sample over Whatman GF/B filters. Filters were rinsed with 7.5 ml ice-cold distilled water, and transferred to scintillation vials. Protosol (1.5 ml) was added to solubilize the tissue, and glacial acetic acid (0.068 ml) and Ready-Solv EP (10 ml) were added. Radioactivity was estimated as described above.

Association dissociation rates, binding studies

A 70 ml incubation mixture of HEPES buffer, membrane suspension (0.15 mg protein/ml), and D-[^{3}H]glucose (final concentration 15.2 nM) was incubated at 37° and stirred intermittently. Duplicate 1 ml fractions were removed at 30 sec, 1 min, and at 1 min intervals for 10 min, and then at 2 to 5 min intervals for an additional 15 min, and filtered over Whatman GF/B filters. Radioactivity estimated in these samples was used to calculate the fractional rate of association. After 20.5 min, unlabelled D-glucose (final concentration 50 µM) was added to a remaining fraction of the mixture and at 30 sec, 1 min, and at 1 min intervals for 10 min, then at 2 to 5 min intervals for another 15 min, duplicate 1 ml fractions were removed and filtered. Radioactivity estimated in these samples was used to calculate the fractional rate of dissociation.

Protein determination

Tissue protein was measured by Bradford's method (1976), with bovine serum albumin (BSA) as the standard. A one-hundred microliter aliquot of sample in HEPES buffer and 5 ml of BioRad dye reagent concentrate (diluted 1:5 parts by volume with distilled water) were added to glass tubes, and the tubes were vortexed briefly. After 10 to 50 min, the optical density at 595 nm (OD_{595}) of the samples was determined with a Sargent-Welsh 6-550 UV/VIS Spectrophotometer. Denatured protein from ATP studies was measured similarly, but samples and standards were in a solution of 100 mM HCl in methanol, and all samples were vortexed to resuspend denatured protein immediately before reading OD_{595}. Protein values for unknowns were based on an experimentally determined standard curve using concentrations of 0 to 100 µg BSA per 0.1 ml; a typical

standard curve is shown in Figure 10.

Data Analysis

The Hewlett Packard 9815A calculator with prerecorded tape cartridge packages was used to evaluate the data by one way analysis of variance or by unpaired t-test. When data showed statistical significance at $P < 0.05$, data were further analyzed by the Newman Keuls Multiple Comparison test (Zivin and Bartko, 1976).

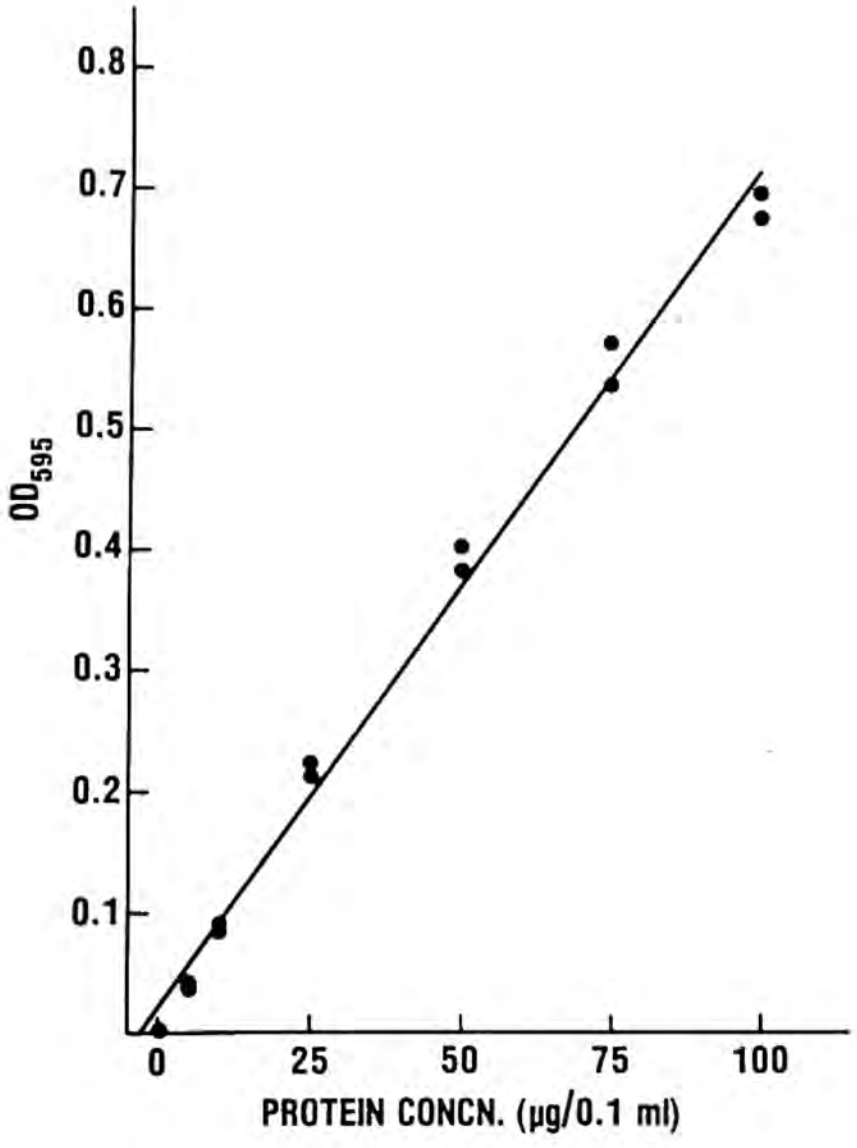

Fig. 10. Standard curve for protein measurement. Each point represents a single sample. Line was estimated by linear regression.

RESULTS

[^{3}H]2-Deoxyglucose Uptake by Slices of Cerebral Cortex

Initial experiments using rat cerebral cortex slices were done to develop a valid *in vitro* model of glucose transport by brain. Brain slices were incubated in the presence of radiolabelled 2-deoxyglucose or equimolar radiolabelled L-glucose, and after incubation, the tissue was filtered, rinsed, solubilized, and radioactivity within the tissue was estimated. The radioactivity present in each sample was used to calculate the amount of [^{3}H]hexose transported according to the following equation:

$$\frac{\text{tritium obtained}}{\text{mg wet tissue wt}} \times \frac{\text{nmoles hexose added}}{\text{total tritium added}} = \frac{\text{nmoles hexose taken up}}{\text{mg wet tissue weight}}$$

and results were expressed as nmoles taken up as a function of tissue weight. L-[^{3}H]glucose taken up by slices was used as an estimate of space available for glucose diffusion. From the amount of L-[^{3}H]glucose taken up by the slices, I calculated that this hexose was distributed to a space equal to 80 to 90% of the wet tissue weight, a space essentially equivalent to cell water. The variable of interest, net [^{3}H]2DG uptake was defined as the difference between [^{3}H]2DG uptake by slices and L-[^{3}H]glucose uptake by slices in parallel samples. In most cases, except when noted otherwise in the Figure Legends, the mean L-[^{3}H]glucose uptake value for a particular treatment group was subtracted from each individual [^{3}H]2DG uptake value for that treatment group to obtain net [^{3}H]2DG uptake values. The concentration of radiolabelled hexose routinely used in these transport studies was 0.5 mM. As shown in Figure 11, un-

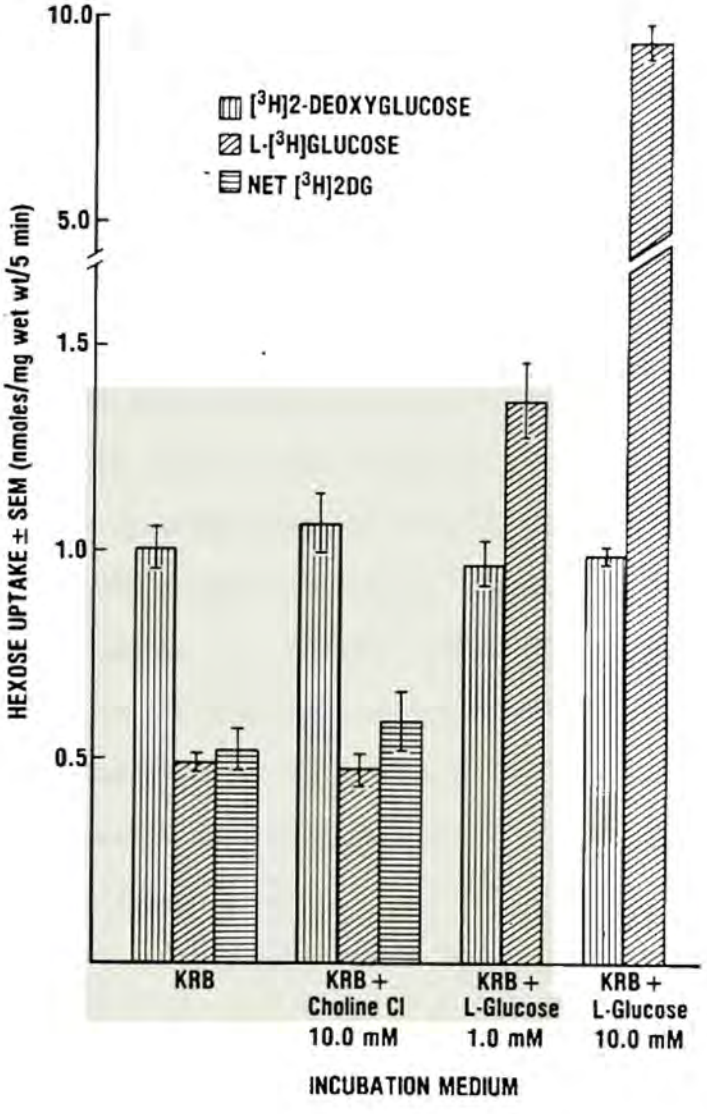

Fig. 11. The effect of L-glucose and choline chloride on hexose uptake by slices of cerebral cortex. Slices were incubated in KRB or in KRB containing 10.0 mM choline chloride, or 1.0 mM L-glucose and 9.0 mM choline chloride, or 10.0 mM L-glucose. Incubation was at 37° for 5 min. Each value is the mean ± SEM of four samples. (Modified from Gold and Kyle, 1981).

der these conditions, the ratio of [^{3}H]2DG uptake to L-[^{3}H]glucose uptake was approximately 2 to 1. This sample to blank ratio was considered adequate for uptake studies. Also shown in this Figure is the lack of effect on [^{3}H]2DG uptake of small changes in osmolarity which would be introduced in kinetic studies, for example, where the hexose concentration was a variable. These changes in osmolarity were effected by adding 10 mM choline chloride, 1 mM L-glucose and 9 mM choline chloride, or 10 mM L-glucose to the incubation medium. Furthermore neither choline chloride nor L-glucose significantly affected the transport of [^{3}H]2DG. L-[^{3}H]glucose uptake by slices was not affected by the addition of choline chloride to the incubation medium. Increasing the concentration of L-glucose in the medium did, however, affect the uptake of L-[^{3}H]glucose. The increase in L-[^{3}H]glucose uptake by slices incubated in the presence of increasing concentrations of L-glucose was directly proportional to the increase in the concentration of L-glucose, and this is consistent with a diffusion process.

Because the kinetic properties of uptake systems should reflect the initial rate of the uptake and not the rate at equilibrium, hexose uptake was studied as a function of incubation time. This study was done to determine the incubation times which best reflected initial rate, and results are shown in Figure 12. [^{3}H]2-Deoxyglucose uptake was apparently linear for 10 min, but the time course if extrapolated did not pass through zero. Uptake of L-[^{3}H]glucose appeared to saturate within 1 min, and net [^{3}H]2DG uptake was linear for at least 10 min. Although a 5 min incubation period was used in preliminary studies, the incubation period was shortened to 2 min for all experiments subsequent to this study. While the time course was linear through at least 10 min, the 2 min incu-

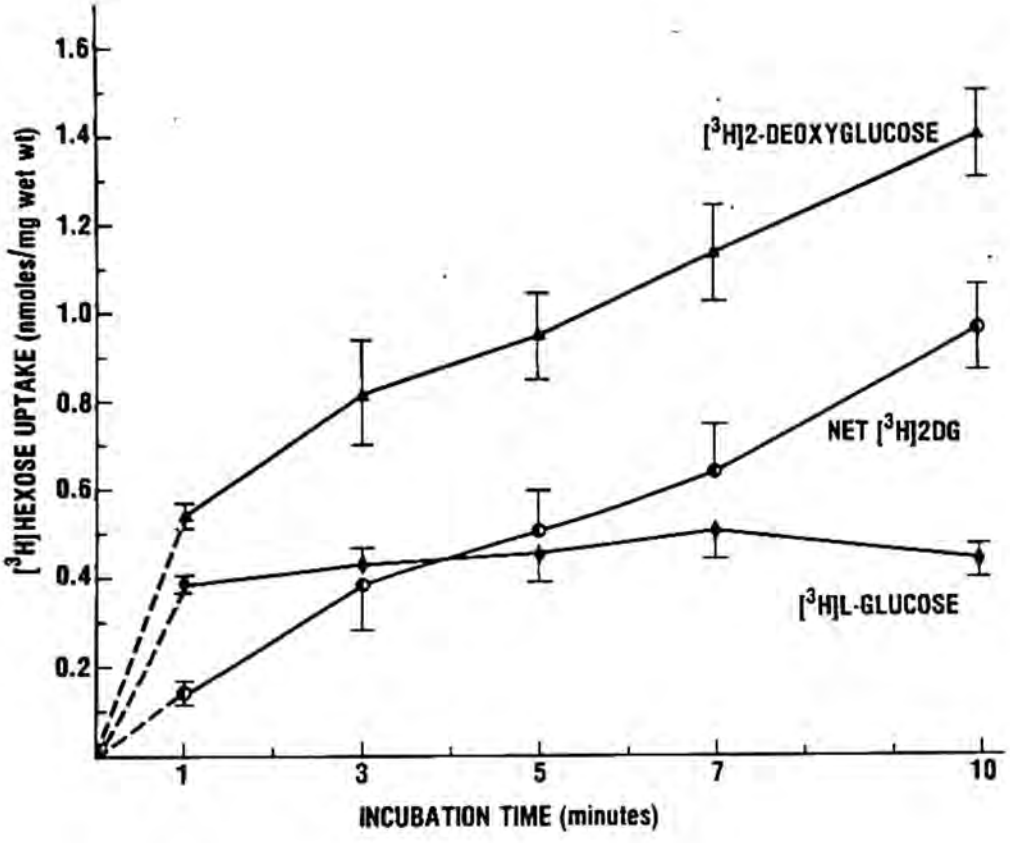

Fig. 12. Hexose uptake as a function of incubation time. Incubation temperature was 25°. Each value is the mean ± SEM of four samples. (From Gold and Kyle, 1981).

bation time was chosen to be certain I was studying initial rates of glucose uptake. The incubation time used for each experiment is noted in the Figure Legend.

Kinetics of [^{3}H]hexose uptake were studied to determine the affinity of the uptake system in the brain slice model for [^{3}H]2DG. Results from this study are shown in Figure 13. As illustrated in the Inset, [^{3}H]2DG uptake appeared to saturate, while L-[^{3}H]glucose uptake did not appear to saturate at the substrate concentrations used. The concentration of [^{3}H]2DG, estimated from the double reciprocal plot shown in the Main Figure, at which net [^{3}H]2DG transport was half maximum (K_m) was 1.85 mM. This is much lower than the K_m for D-glucose uptake by human erythrocytes which in Lehninger's text is reported to be 6.2 mM (1975). Maximum velocity (V_{max}) for net [^{3}H]2DG uptake by brain slices was estimated to be 1.43 nmoles/mg wet wt/2 min.

To optimize further the <u>in vitro</u> assay conditions for uptake studies, the effect of incubation temperature on net [^{3}H]2DG uptake by slices was studied. As shown in Figure 14, when temperature was increased above 15°, net [^{3}H]2DG uptake increased, and the relationship between temperature and uptake was linear from 15° to 25°. A temperature coefficient, Q_{10}, equal to 1.87 from 15° to 25° was estimated by dividing the value obtained for net [^{3}H]2DG transport at 25° by the value obtained for net transport at 15°. There was an inflection point at 25° and a lower Q_{10} from 25° to 37°. A $Q_{10} \cong 1.4$ usually represents a passive diffusion process while a $Q_{10} \cong 2.0$ may represent an enzyme catalyzed reaction or an active process (Christensen, 1975). An activation energy of 11.4 kcal/mole was derived for $Q_{10} = 1.87$ according to the following equation: $Q_{10} = e^{0.055A}$, where A is activation energy in kcal/mole (Stein, 1967).

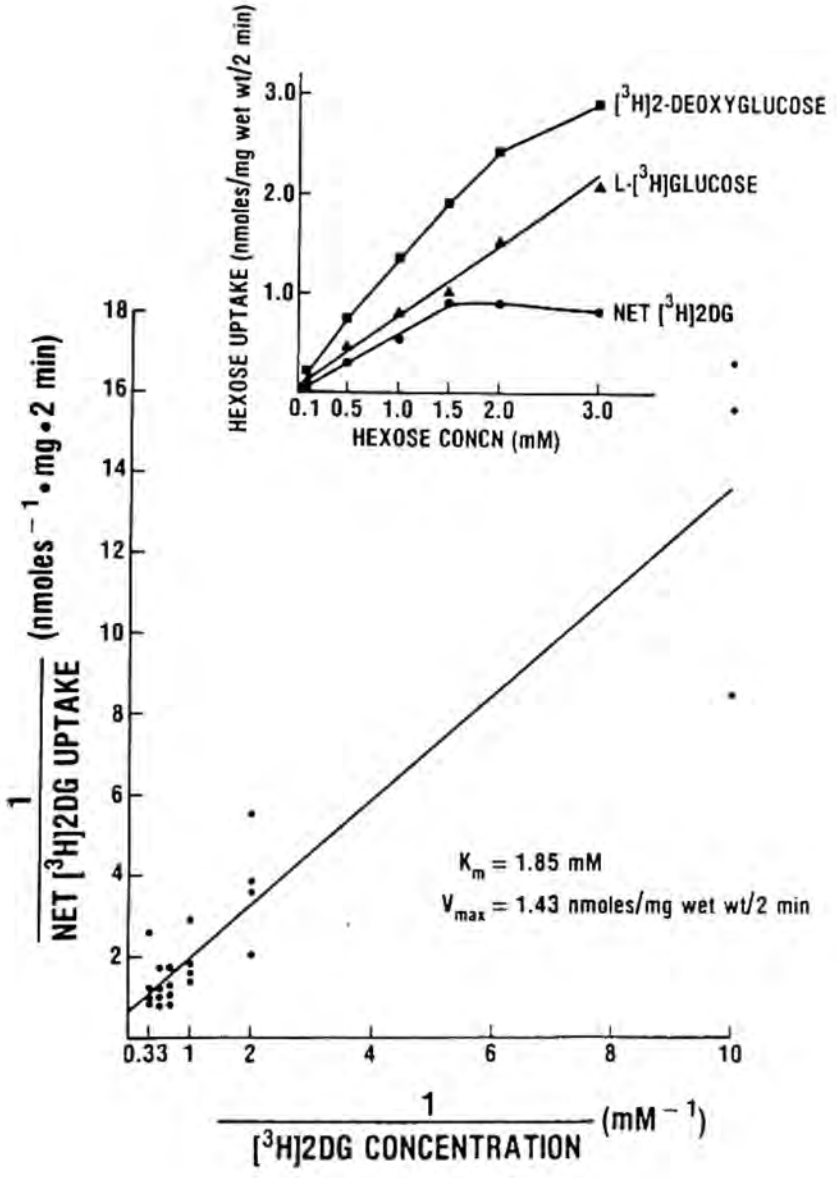

Fig. 13. Inset- Hexose uptake by slices as a function of hexose concentration. Slices were incubated at 25° for 2 min. Each value is the mean of three or four samples. Main Figure- Double reciprocal plot of net [^{3}H]2DG transport. Points are the individual values used to obtain the net means in the Inset. The line was estimated by linear regression.

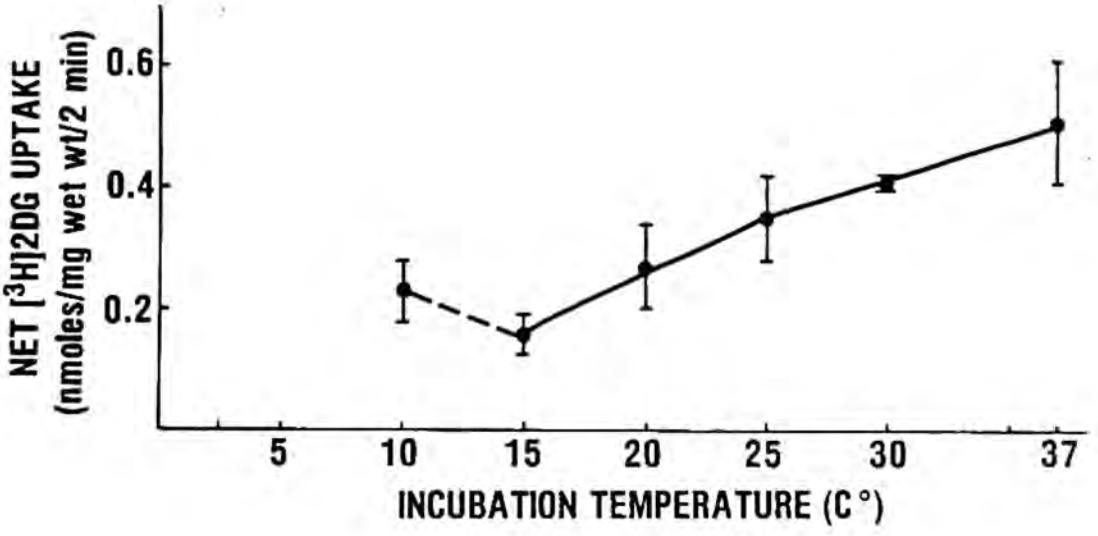

Fig. 14. Net [^{3}H]2-deoxyglucose transport by slices of cerebral cortex as a function of incubation temperature. Slices were incubated for 2 min. Each value is the mean ± SEM of four samples. (Modified from Kyle-Lillegard and Gold, 1983).

L-[^{3}H]glucose uptake was unaffected by the incubation temperatures studied; these results are not shown. After this study, all incubations were done at 25° instead of 37°.

Hexose is cotransported with sodium in the intestine and in the kidney (Lehninger, 1975). To see whether sodium, or any other ions played a role in [^{3}H]2DG transport by brain slices, a series of experiments were designed to study the ionic requirements of net [^{3}H]2DG transport. To facilitate the deletion of specific ions from the incubation medium to study ionic requirements of hexose uptake by brain slices, I used Krebs-Ringer phosphate medium instead of KRB. Net [^{3}H]2DG uptake was not changed significantly when the KRB incubation medium was substituted with Krebs-Ringer-phosphate medium (KRP) which contained 122 mM NaCl; 5 mM KCl; 1.3 mM $CaCl_2$; 1.2 mM $MgSO_4$; and 15.8 mM Na_2HPO_4 titrated to pH 7.4 with NaH_2PO_4, and was bubbled with 95% O_2/5% CO_2 before it was added to the vials. The final pH of this solution was approximately 6.5. These results are shown in Figure 15, and were qualitatively similar for both concentrations of [^{3}H]hexose tested. Also apparent in this Figure is the much lower ratio of [^{3}H]2DG uptake to L-[^{3}H]glucose uptake at the higher hexose concentration tested. Because there was no apparent difference between [^{3}H]2DG transport by slices incubated in KRB compared to transport by slices incubated in KRP, these media were both used in the ionic requirement studies, the results of which are shown in Table 1. Net transport was unaffected when Na^+ or K^+ were singularly deleted and replaced with equimolar choline. Net [^{3}H]2DG uptake was not significantly changed when Mg^{2+} or Ca^{2+} was deleted, when Ca^{2+} was deleted and EGTA was added, or when Cl^- salts were replaced by acetate salts of the metals. Also, there was no effect on net [^{3}H]2DG transport when $MgSO_4$

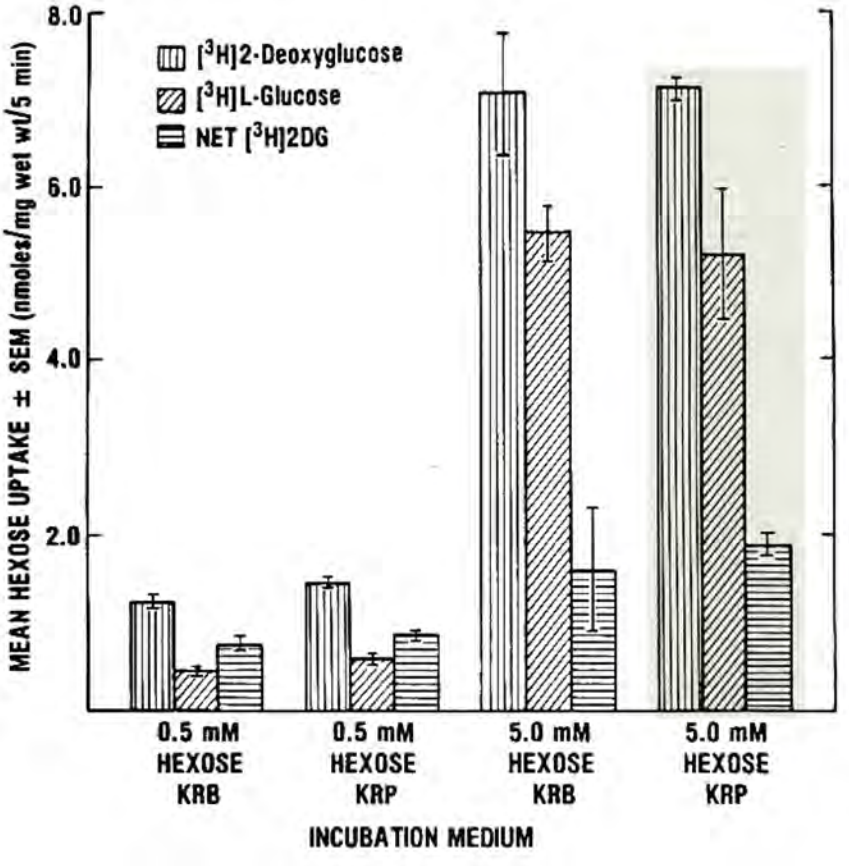

Fig. 15. The effect of phosphate and bicarbonate incubation media on hexose uptake by slices of cerebral cortex. Incubation was at 37° for 5 min. Each value is the mean ± SEM of four samples. (From Gold and Kyle, 1981).

TABLE I

THE EFFECT OF INCUBATION MEDIA DEFICIENT IN VARIOUS IONS ON NET [^{3}H]2-DEOXYGLUCOSE TRANSPORT BY SLICES OF RAT CEREBRAL CORTEX

INCUBATION MEDIUM	n	MEAN % OF CONTROL ± SEM
Control* KRB and KRP	23	100
Na^+-free KRP	4	102.6 ± 0.05
K^+-free KRP	4	92.7 ± 0.08
Mg^{2+}-free KRB	3	93.6 ± 0.02
Ca^{2+}-free KRB	4	90.2 ± 0.07
Ca^{2+}-free KRB + 50 µM EGTA	4	109.0 ± 0.02
Cl^--free	4	126.7 ± 0.15

* Control values for each day were taken as the mean of three or four samples and arbitrarily designated as 100. Incubations were at 37° for 5 min. The grand mean ± SEM for all controls was 0.657 ± 0.037 nmoles/mg wet weight/5 min. (From Gold and Kyle, 1981).

was replaced with $MgCl_2$ or with $MnSO_4$ (data not shown). These data indicate that hexose is not cotransported with sodium in brain slices, and there was no apparent effect of the deletion of any other single ion tested on net transport of [^{3}H]2DG.

Up to this point, I had shown that net [^{3}H]2DG transport by slices of rat cerebral cortex was saturable, temperature dependent, not sensitive to small changes in osmolarity, and apparently not dependent on the presence of any particular ion present in the KRB medium. The next studies were planned to compare some of the characteristics of net [^{3}H]2DG transport by brain slices with some of the known characteristics of glucose transport by other systems.

While the hexose uptake system is selective for D-glucose, a number of other sugars are also transported by known D-glucose carriers. Two hexose analogues which are commonly used to study transport are 2DG (the analogue used here) and 3-O-methylglucose (3-O-MG). The ability of these hexoses to inhibit the transport of [^{3}H]2DG by brain slices was studied. Because the 2DG uptake model in brain slices should represent D-glucose uptake, the ability of D-glucose to inhibit net [^{3}H]2DG uptake was also studied. As shown in Figure 16, net [^{3}H]2DG transport was inhibited by 2-deoxyglucose and by D-glucose. The concentration of 2-deoxyglucose which caused a 50% inhibition of transport (IC50) was estimated from these data to be approximately 0.6 mM. The IC50 for inhibition of net [^{3}H]2DG transport by D-glucose was estimated to be approximately 0.9 mM. The IC50 for transport inhibition by 3-O-methylglucose could not be estimated from this graph. These results show that 2DG and D-glucose hexoses which are known to be transported by the glucose carrier in other tissues, inhibit [^{3}H]2DG uptake by brain slices. The analogue 3-O-MG,

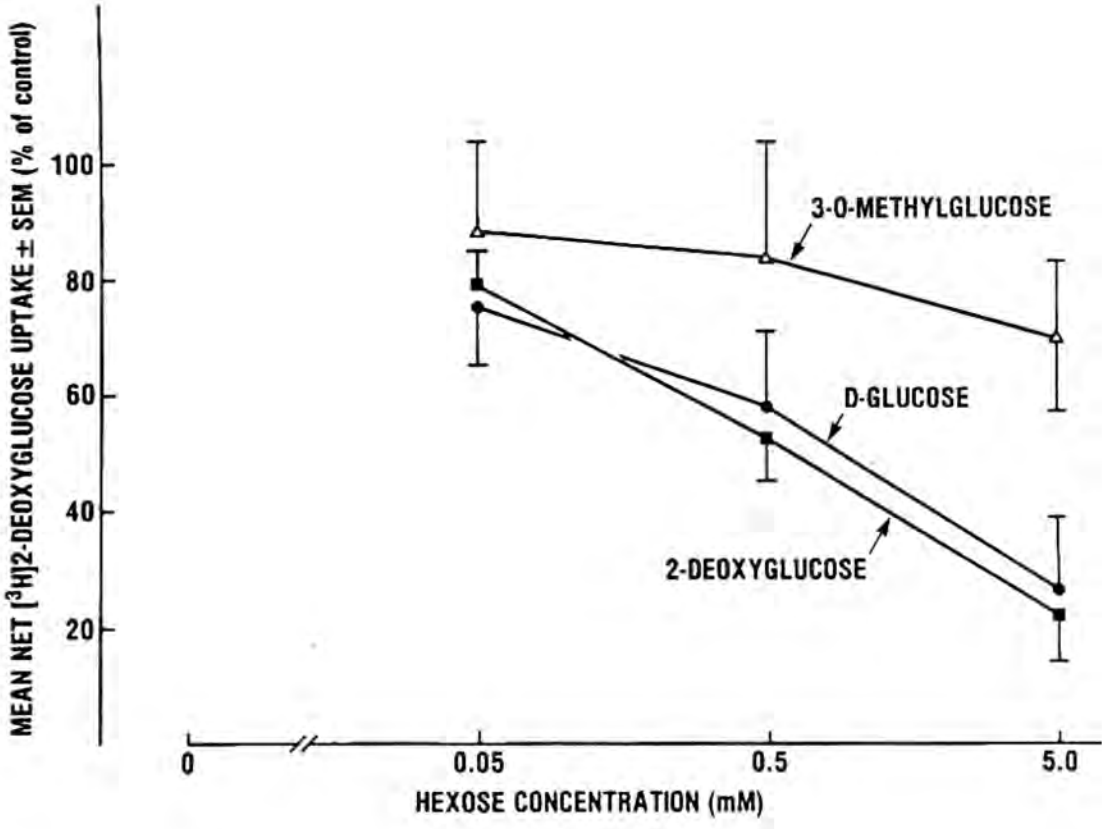

Fig. 16. Net [^{3}H]2DG transport by slices of cerebral cortex, inhibition by other hexoses. Slices were incubated in KRB containing hexoses in the concentrations shown. Incubation was at 25° for 2 min. Values are means ± SEM of four samples.

while also inhibitory, was much less so when compared to D-glucose or to 2DG.

Inhibition of hexose transport by phlorizin or phloretin has been described for other tissues, so the effect of these compounds on the transport of [^{3}H]2DG by brain slices was determined. Net [^{3}H]2DG transport was not inhibited in the presence of phlorizin or phloretin. These compounds are known to inhibit glucose transport by the small intestine (Alvarado and Crane, 1962) and by erythrocytes (LeFevre and Marshall, 1959), respectively. These results are shown in Figure 17, and suggest that the hexose uptake system in brain slices may be different from uptake by erythrocytes and from uptake by intestine.

In an active transport system, the solute is transported at the expense of metabolic energy. To test whether brain slice hexose uptake was energy dependent, a preliminary study was first designed to test whether net [^{3}H]2DG transport was sensitive to mitochondrial poisons. Results from this study are shown in Figure 18. Slices were incubated in the presence of 2,4-dinitrophenol (DNP) an uncoupler of oxidative phosphorylation, or in the presence of sodium azide (NaN_3) a cytochrome oxidase inhibitor. Transport in the presence of either of these compounds appeared to decrease, although inhibition of transport was statistically significant by one way analysis of variance only in the presence of 1.0 mM DNP. Results from this study led me to hypothesize that net [^{3}H]2DG transport may be partly dependent on mitochondrial energy.

To test this hypothesis further, I studied net [^{3}H]2DG uptake by slices which were preincubated under conditions designed to alter intracellular adenosine 5'-triphosphate (ATP) stores. Results from these studies are shown in Figure 19. Data in Panel A are from slices which were

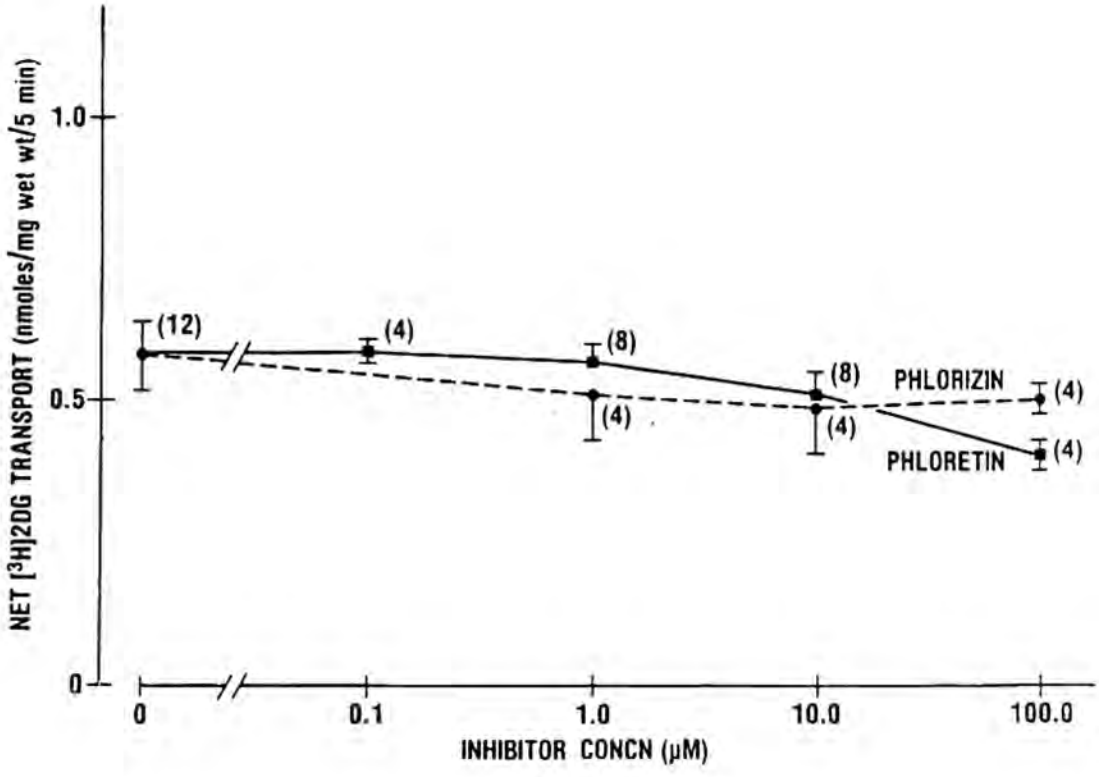

Fig. 17. The effect of phlorizin and phloretin on net [^{3}H]2-deoxyglucose transport by slices of cerebral cortex. Slices were incubated in KRB containing inhibitor at the concentrations shown. Incubation was at 25° for 5 min. Values are means + SEM of the number of samples indicated. (Modified from Kyle-Lillegard and Gold, 1983).

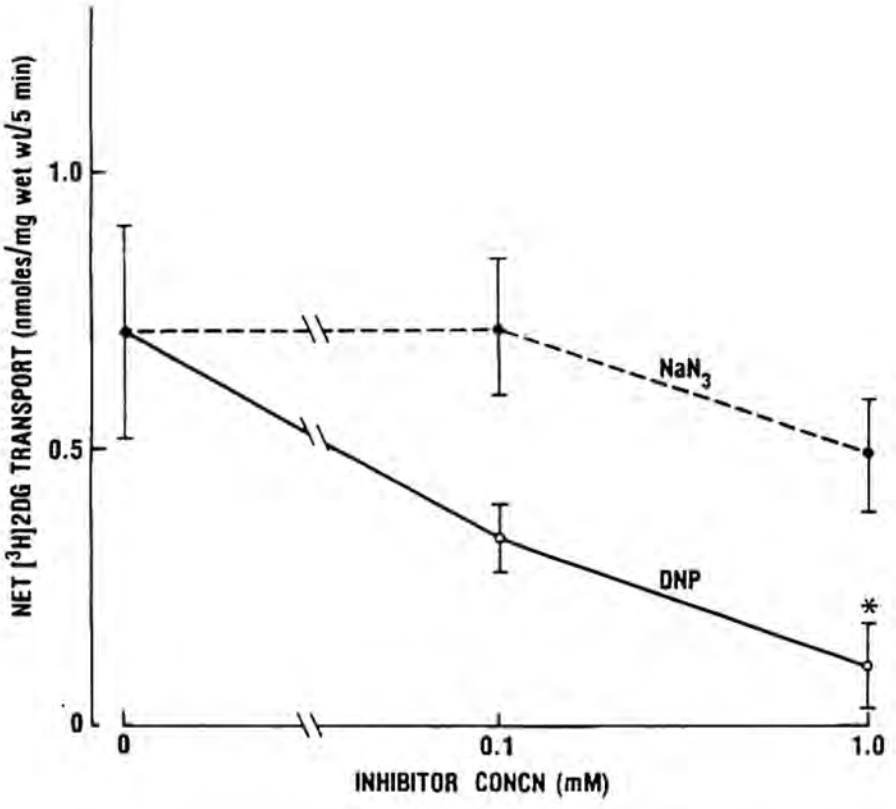

Fig. 18. The effect of sodium azide and 2,4-dinitrophenol on net [^{3}H]2-deoxyglucose transport by slices of cerebral cortex. Incubation was at 37° for 5 min. Each value is the mean ± SEM of four samples. $^*P < 0.05$ as compared to control. (From Gold and Kyle, 1981).

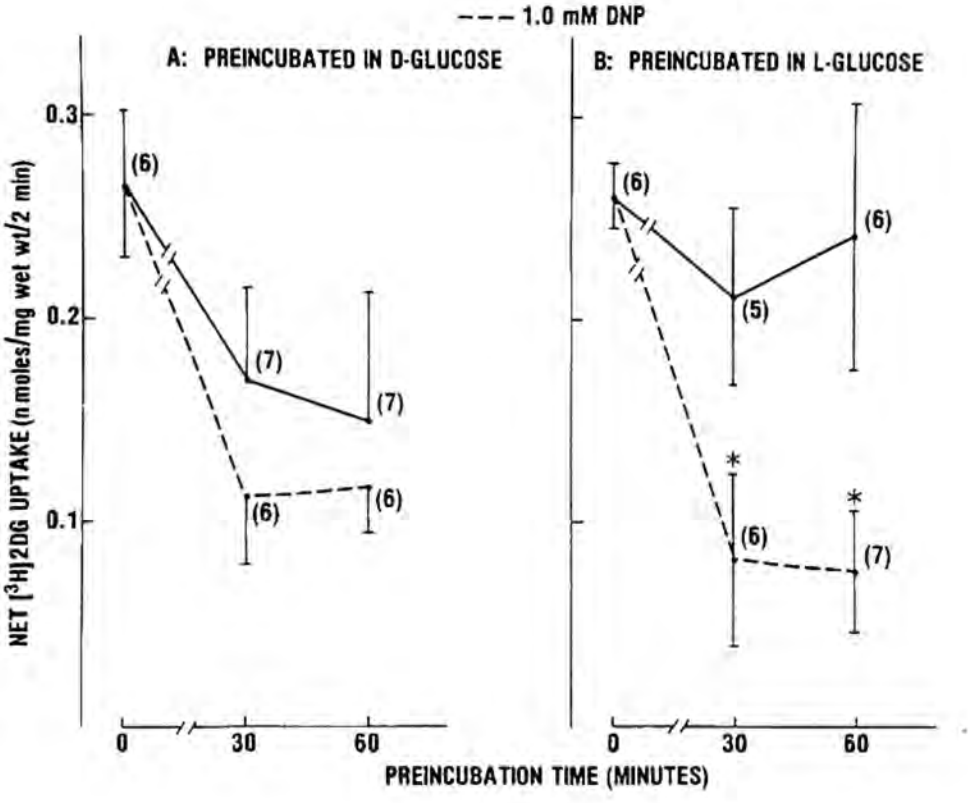

Fig. 19. Hexose transport by slices of cerebral cortex as a function of preincubation time: the effect of D- or L-glucose and DNP. Slices were preincubated in KRB containing 10.4 mM D-glucose (A) or 10.4 mM L-glucose (B) with or without DNP. Incubation was at 25° for 2 min. Values are means ± SEM of the number of samples indicated. Blank values were single parallel L-[^{3}H]-glucose samples. *$P < 0.05$ as compared to zero pre-incubation time. (From Kyle-Lillegard and Gold, 1983).

preincubated in KRB containing 10.4 mM D-glucose (the concentration of D-glucose present in unmodified KRB medium) with or without 1.0 mM DNP for the times indicated. Data in Panel B are from slices preincubated in KRB containing 10.4 mM L-glucose, which is not a substrate for glycolysis, with or without DNP. Also included in this design were slices which were not preincubated. Net [^{3}H]2DG uptake appeared to decrease after preincubation in D-glucose, and DNP appeared to decrease net uptake further. These apparent decreases in net transport were not statistically significant by one-way analysis of variance ($F_{4,27} = 1.79$, $P = 0.16$), and this was probably due to large within-group error. Unpaired t-tests were used in a separate analysis to compare net [^{3}H]2DG transport by slices preincubated with D-glucose with net [^{3}H]2DG transport by slices preincubated for an equivalent time with D-glucose and DNP, and there were no significant differences at 30 or 60 min. Net [^{3}H]2DG uptake was unaffected after preincubation with L-glucose for 30 or 60 min, but net uptake was significantly decreased after preincubation with L-glucose and DNP. Unpaired t-tests showed that net [^{3}H]2DG uptake by slices exposed to L-glucose alone was significantly different from net uptake by slices exposed to L-glucose and DNP at both 30 and 60 min. From these studies, extended preincubation in the presence of D-glucose appeared to decrease subsequent net [^{3}H]2DG transport while extended preincubation in the presence of L-glucose, which is not a substrate for glycolysis and is apparently not metabolized by the cell, was without effect on subsequent transport. Preincubation in the presence of DNP inhibited subsequent transport of [^{3}H]2DG.

To examine further the cellular energetics after preincubating slices with or without metabolic substrates, the ATP levels were measured in slices which had been preincubated in conditions similar to those

described above. Data are shown in Figure 20. ATP levels measured in slices which had been preincubated for 60 min in KRB containing 10.4 mM D-glucose were unchanged from levels in slices which had not been preincubated. Preincubation for 60 min in KRB containing 10.4 mM L-glucose resulted in a 65% decrease in ATP levels as compared to levels in slices which had not been preincubated. ATP levels in slices exposed to DNP during preincubation or during incubation (no preincubation) were decreased by at least 75% compared to slices which were not preincubated, and not exposed to DNP. There is, then, an apparent correlation between reduced ATP levels after preincubation with DNP and reduced transport subsequent to preincubation with DNP. The correlation between ATP levels and the ability of the slices to transport [^{3}H]2DG after preincubation with D-glucose or with L-glucose is unclear.

Because of this lack of convincing correlation between ATP levels and subsequent ability to transport [^{3}H]2DG after extended preincubation, further studies were done to examine [^{3}H]2DG uptake by slices preincubated for long periods in the absence of an exogenous metabolic substrate. While testing the effects of extended preincubation on [^{3}H]2DG uptake by slices it was important to be sure that slices were well equilibrated before subjecting them to an hour in the absence of metabolic substrate. After 30 min in KRB containing glucose brain slices repleted many of the endogenous substances depleted immediately after decapitation (Bachelard, 1976). The preincubation assay for uptake studies was therefore modified to include a 30 min preincubation period for all slices. Slices were preincubated at 25° in an atmosphere of O_2/CO_2 in KRB medium which contained 10.4 mM D-glucose. This preincubation period was included in all subsequent studies as noted in the Figure Legends. In these experiments,

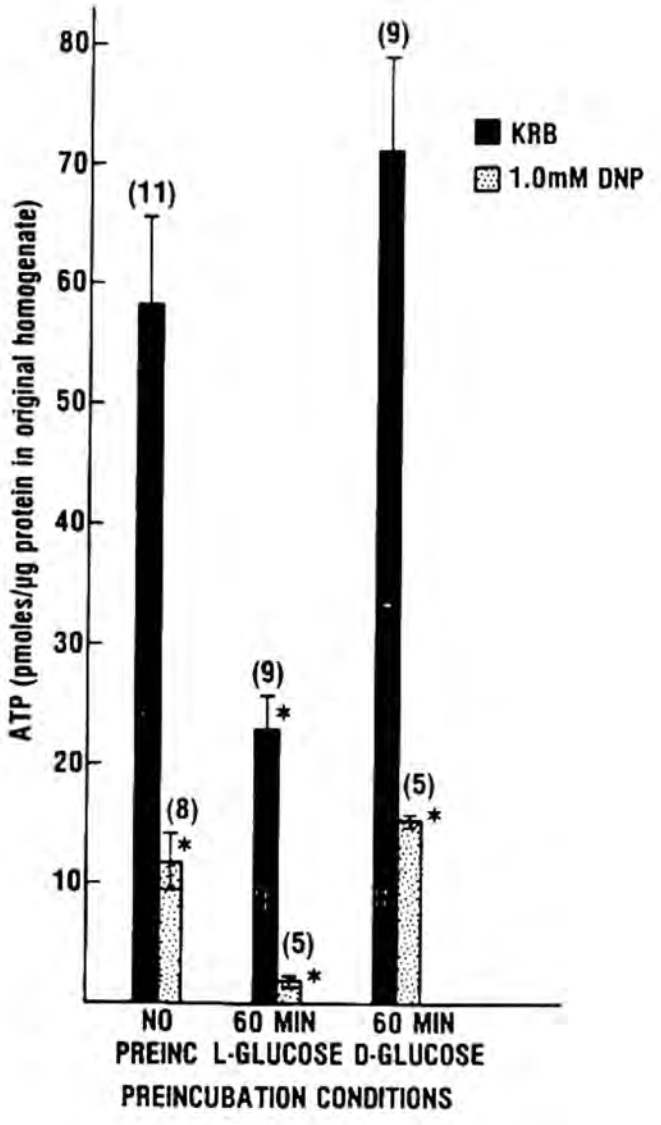

Fig. 20. ATP levels in slices of cerebral cortex after preincubation in D- or L-glucose and DNP. Slices were preincubated in KRB containing 10.4 mM L-glucose or 10.4 mM D-glucose with or without DNP at 25°. Values are means ± SEM of duplicate ATP measurements for the number of samples indicated. Results are not corrected for recovery of ATP from tissues, calculated to be 28%. *$P < 0.05$ as compared to no preincubation or to preincubation in D-glucose. (From Kyle-Lillegard and Gold, 1983).

slices were preincubated for selected times in KRB containing 10.4 mM choline chloride (Figure 21, A) or 10.4 mM L-glucose (Figure 21, B), and the subsequent ability of these slices to transport [^{3}H]2DG was compared to that of slices which had been preincubated with KRB containing 10.4 mM D-glucose for an equivalent time. At each time tested, net [^{3}H]2DG uptake by slices preincubated in KRB containing D-glucose was not different by one way analysis of variance from net [^{3}H]2DG uptake by slices which were not preincubated. Also shown in this Figure, with a single exception (Panel A, 30 min), is the significantly increased net transport by slices preincubated without D-glucose (but in choline chloride or L-glucose to maintain tonicity) as compared to net transport by slices preincubated for the same period of time with D-glucose. In these studies, preincubation with L-glucose caused an increase in subsequent transport of [^{3}H]2DG and preincubation with D-glucose caused no change in subsequent transport. These results are somewhat different from those obtained in a similar study (Figure 19) when preincubation with L-glucose caused no change in subsequent transport and D-glucose caused an apparent but not statistically significant decrease in subsequent net transport of [^{3}H]2DG. These differences were probably a result of the assay modification which was introduced in the later study.

Figure 22 shows results from another extended preincubation study in which net [^{3}H]2DG transport by slices which were not preincubated was compared to net transport by slices which were preincubated for 60 min in KRB containing 10.4 mM D-glucose, 10.4 mM 3-O-methylglucose, 10.4 mM 2-deoxyglucose, or 2.0 mM D-glucose. The 10.4 mM concentration was chosen because it is the concentration of D-glucose normally found in unmodified KRB media; the lower concentration of D-glucose was chosen

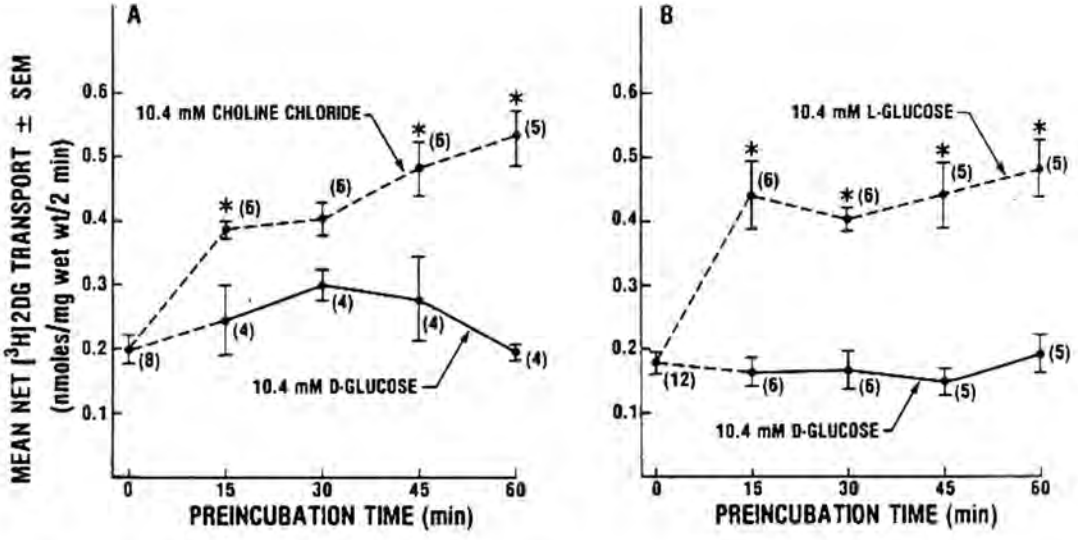

Fig. 21. The effect of extended preincubation with or without D-glucose on subsequent net [^{3}H]2DG transport by cerebral cortex slices. All slices were preincubated in KRB containing 10.4 mM D-glucose for 30 min, and then slices were preincubated in KRB containing 10.4 mM choline chloride (A) or 10.4 mM L-glucose (B) or 10.4 mM D-glucose for the times indicated. Incubation was at 25° for 2 min. Values are means ± SEM of the number of samples shown. *$P < 0.05$ as compared to 0 min and to the corresponding sample preincubated in D-glucose.

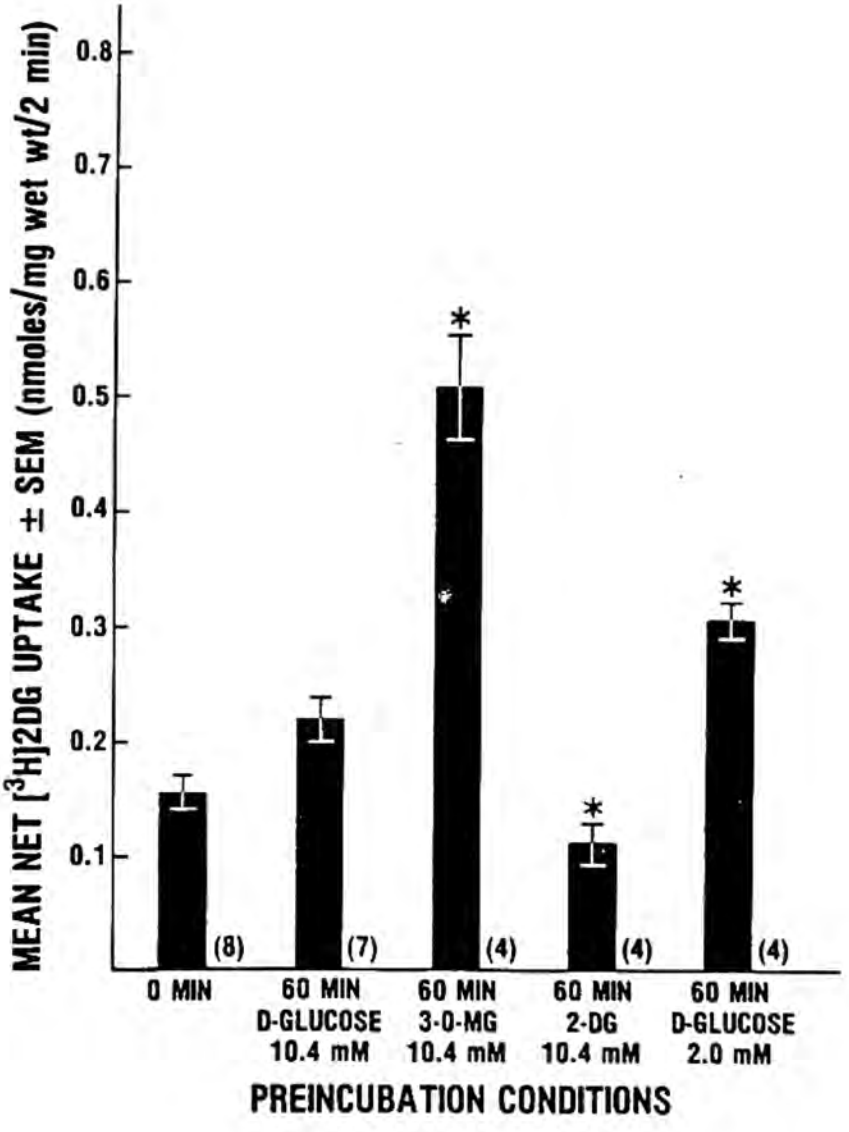

Fig. 22. The effect of extended preincubation with selected hexoses on subsequent net [^{3}H]2DG transport by slices. All slices were preincubated for 30 min in KRB containing 10.4 mM D-glucose and then slices were preincubated for an additional 60 min in KRB containing hexoses in the concentrations shown. Incubations were at 25° for 2 min. Values are means ± SEM of the number of samples indicated. *$P < 0.05$ as compared to 0 min or to 60 min in 10.4 mM D-glucose. Net uptake after preincubation in 10.4 mM 2-DG was only different from net uptake after 10.4 mM D-glucose.

because at this plasma concentration of glucose, symptoms of hypoglycemia appear (Bachelard, 1975). In agreement with an earlier experiment (Figure 21), net transport by slices which were not preincubated was not different from net transport by slices preincubated for 60 min in 10.4 mM D-glucose. Net [^{3}H]2DG uptake was increased 3-fold after slices were exposed to 10.4 mM 3-O-MG as compared to net uptake by slices which were not preincubated; net uptake by slices exposed to 2.0 mM D-glucose was increased 2-fold when compared to uptake by slices which were not preincubated. The ability of slices to transport [^{3}H]2DG after preincubation with 10.4 mM 2DG was unchanged when compared to slices which were not preincubated, but was slightly decreased when compared to slices preincubated with 10.4 mM D-glucose. In another study, slices were preincubated for 60 min in the presence of 10.4 mM sodium pyruvate, and transport of [^{3}H]2DG by these slices was significantly increased when compared to transport by slices which had been preincubated for 60 min in 10.4 mM D-glucose or when compared to slices which had not been preincubated (data not shown). In summary, these studies suggest that preincubation of slices in the absence of a sufficient substrate for hexokinase (choline chloride, L-glucose, 3-O-MG, 2 mM D-glucose, sodium pyruvate) activated net [^{3}H]2DG uptake and that preincubation of slices in the presence of an adequate substrate (10.4 mM D-glucose, 2DG) in some way prevented the activation.

A group of experiments was performed to determine if these results could be explained by changes in hexokinase activity rather than on uptake alone. In the slice preparation hexokinase catalyzes the phosphorylation of 2-deoxyglucose to 2-deoxyglucose-6-phosphate at the expense of ATP. To study this hexokinase step, I first tested the effect of incubation time on the amount of tritium recovered from slices, which coeluted with

authentic [^{14}C]glucose-6-phosphate. Data from these experiments are shown in Figure 23. The percent of recovered [^{3}H] in the acid fraction, which represented tritium coeluting with phosphorylated hexose standards, increased as incubation time in the presence of [^{3}H]2DG was increased. The relationship between percent recovered tritium in the HCl fraction and incubation time appeared linear for at least 3 min, and then began to saturate at the longer incubation times studied. The mean percent of recovered tritium eluted with HCl after slices were incubated with L-[^{3}H]-glucose was always less than 3 percent, and did not change as a function of incubation time, consistent with the stereospecificity of hexokinase (Gold and Kyle, 1981). From these results, it was apparent that the phosphorylation reaction catalyzed by hexokinase was dependent on time, and there was always free glucose measurable within the tissue at the incubation times studied.

To determine whether the large increase in net [^{3}H]2DG uptake seen after slices were preincubated without D-glucose (see Figures 21 and 22) was also accompanied by an increase in hexokinase activity, I tested the effect of preincubating brain slices for long periods of time in the absence of an exogenous metabolic substrate on subsequent net [^{3}H]2DG uptake by these slices and on the percent of tritium subsequently recovered from these slices eluted in the HCl fraction. Data from this study are shown in Figure 24. Net transport was decreased slightly after preincubation for 60 min in 10.4 mM D-glucose as compared to transport by slices which were not preincubated (0), but this change was not statistically significant. Subsequent to a 60 min preincubation in 10.4 mM choline chloride, net [^{3}H]2DG transport by slices was increased 3-fold when compared to transport by slices which were preincubated for an equivalent

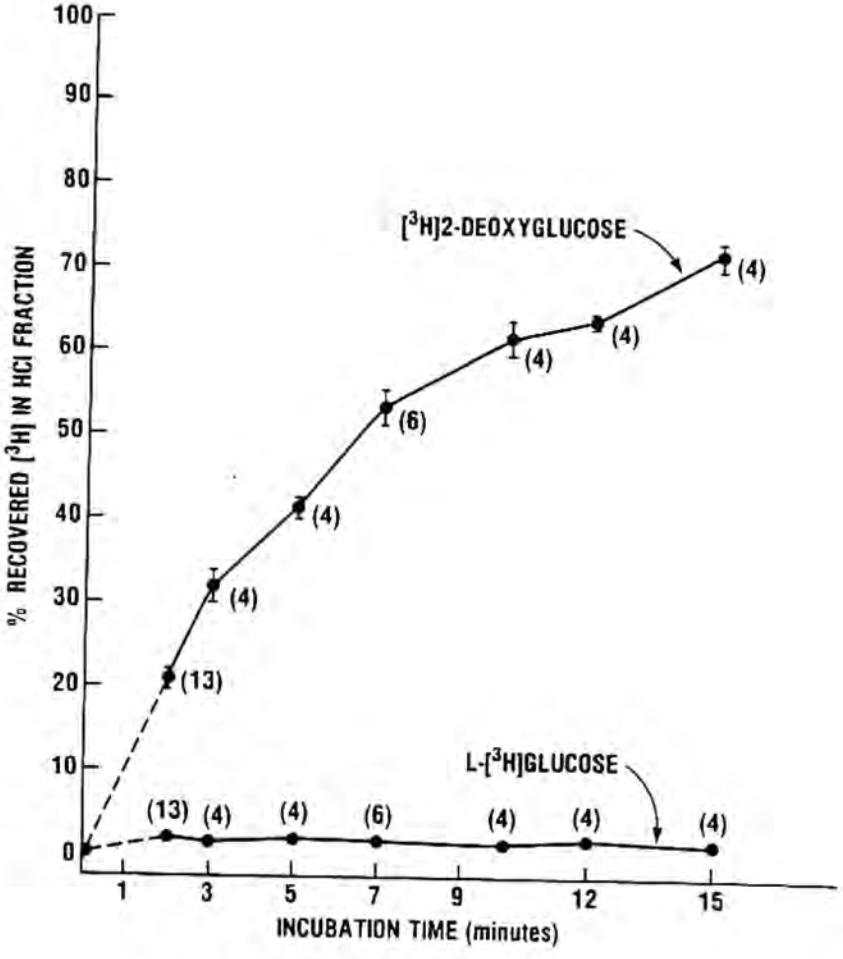

Fig. 23. Percent recovered tritium coeluting with authentic [^{14}C]glucose-6-phosphate as a function of slice incubation time. All slices were preincubated in KRB containing 10.4 mM D-glucose for 30 min and slices were then incubated in KRB containing 10.4 mM choline chloride for the times shown. Incubations were at 25°. Values are means ± SEM of the number of samples indicated. L-[^{3}H]-glucose samples shown are means only, SEMs were always less than 10% of the mean, with the exception of the 5 min point (X = 1.87 ± 0.35).

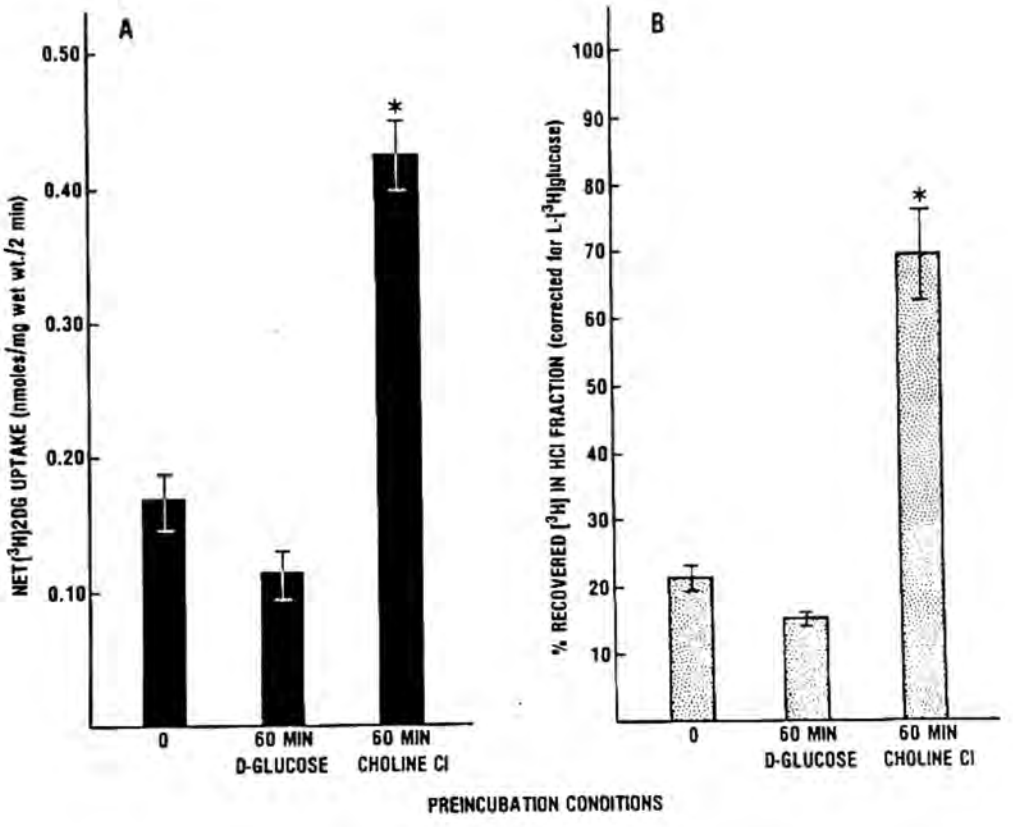

Fig. 24. The effect of long preincubation with or without D-glucose on subsequent net [^{3}H]2DG transport and percent recovered tritium coeluting with [^{14}C]glucose-6-phosphate. All slices were preincubated for 30 min at 25° in KRB containing 10.4 mM D-glucose. Slices were then preincubated for an additional 60 min in KRB containing 10.4 mM D-glucose or in KRB containing 10.4 mM choline chloride. Slices were rinsed and transferred to fresh KRB containing 10.4 mM choline chloride for transport studies. Slices which were not exposed to the 60 min preincubation period were included in the design. Incubations were at 25° for 2 min. Values are means ± SEM of four samples. Values in Panel A were calculated from [^{3}H] estimated after samples were centrifuged and neutralized. Values in Panel B were calculated as the difference between [^{3}H] recovered in the HCl fraction from samples incubated with [^{3}H]2DG and samples incubated with L-[^{3}H]-glucose. *$P < 0.05$ as compared to slices which were not preincubated and to slices preincubated for 60 min with D-glucose.

time in D-glucose or when compared to transport by slices which were not preincubated. The percent recovered tritium eluted with HCl after slices were preincubated for 60 min in 10.4 mM D-glucose was not significantly changed when compared to that from slices which were not preincubated. Consistent with the data in Panel A, after a 60 min exposure to 10.4 mM choline chloride, the percent recovered tritium eluted with HCl was increased when compared to that of slices which were not preincubated, and was almost 5 times greater than the percent recovered tritium eluted with HCl after preincubation in D-glucose. These results show that extended preincubation of slices in the absence of D-glucose causes an increase in both subsequent [^{3}H]2DG uptake and in the subsequent percent of tritium recovered which coelutes with [^{14}C]glucose-6-phosphate.

Sokoloff and coworkers (1977) have shown with autoradiography of [^{14}C]2-deoxyglucose that increased neuronal activity is closely correlated with increased glucose utilization. A final study of the relationship of hexokinase activity to hexose uptake in cerebral cortex slices was to test the effect of a model of increased neuronal activity on net [^{3}H]2DG transport by slices, and on the amount of tritium recovered from these slices which coeluted with glucose-6-phosphate. Results from these studies are shown in Figure 25, Panels A and B. Increased neuronal activity was modeled <u>in vitro</u> by increasing the concentration of extracellular potassium ion in the preincubation medium to 50.2 mM, a concentration known to depolarize neurons (Hillman and McIlwain, 1961). After preincubation for 30 min in KRB containing 10.4 mM D-glucose, brain slices were exposed to this KRB medium containing 50.2 mM KCl and 10.4 mM D-glucose for 5 min (↑K^{+}KRB), and were then rinsed and transferred to fresh KRB medium for transport studies. Slices which were not

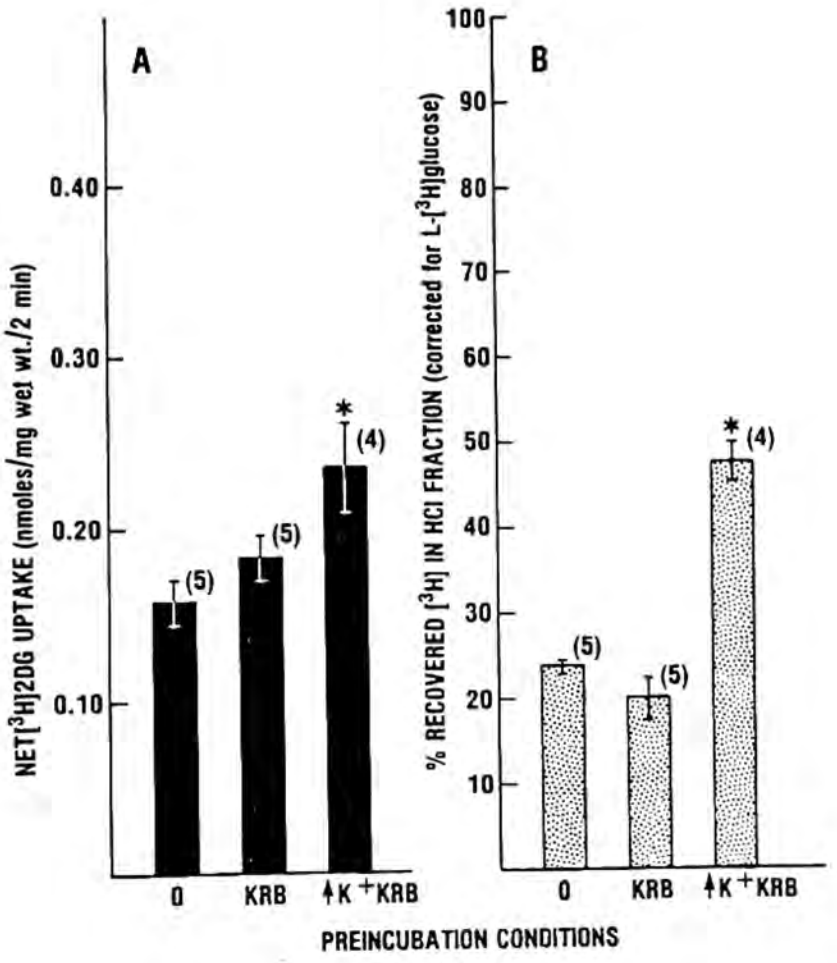

Fig. 25. The effect of potassium depolarization on subsequent net [^{3}H]2DG transport and percent recovered tritium coeluting with [^{14}C]glucose-6-phosphate. All slices were preincubated in KRB containing 10.4 mM D-glucose for 30 min. Slices were then exposed for a 5 min preincubation period to KRB containing 50.2 mM KCl and 10.4 mM D-glucose (↑K$^+$KRB) or to KRB containing 5 mM KCl and 10.4 mM D-glucose (KRB) for 5 min. Slices were rinsed and transferred to fresh KRB containing 10.4 mM choline chloride for transport studies. Slices which were not exposed to the 5 min preincubation period (0) were included in the design. Incubations were at 25° for 2 min. Values are means ± SEM of the number of samples shown. Values in Panel A were calculated from [^{3}H] estimated after samples were centrifuged and neutralized. Values in Panel B were calculated as the difference between [^{3}H] recovered in the HCl fraction from samples incubated with [^{3}H]2DG and samples incubated with L-[^{3}H]glucose. *$P < 0.05$ as compared to 0 (A) or as compared to 0 and KRB (B).

exposed to a 5 min preincubation period in high KCl (0) or which were exposed to a 5 min preincubation period in KRB containing 5 mM KCl and 10.4 mM D-glucose (KRB) were included in the design. As shown in Panel A, net [^{3}H]2DG uptake by slices was increased after preincubation in KRB with 50.2 mM KCl, as compared to slices which had not been preincubated for this 5 min period, or which had been preincubated for 5 min in KRB with 5 mM KCl. Data in Panel B show that the percent of recovered [^{3}H] collected in the HCl fraction was increased 2-fold after a 5 min exposure to high KCl when compared to slices which were not exposed. Thus, this experimental model of increased neuronal activity resulted in an increase in both subsequent net [^{3}H]2DG uptake and the percent of tritium recovered in the HCl fraction. These data suggest that both uptake and phosphorylation are influenced subsequent to increased neuronal activity, but whether this is secondary to increased glycolytic activity or is driving the increased metabolism cannot be deduced.

D-[^{3}H]Glucose Binding to Crude Membrane Fractions Prepared from Rat Cerebral Cortex

Up to this point, I have shown that net [^{3}H]2DG uptake by brain slices is saturable and specifically inhibited by either unlabelled 2DG or by unlabelled D-glucose. Saturability and specific inhibition are two necessary criteria which must be satisfied for solute translocation to be classified as facilitated transport (Lehninger, 1975). In contrast to nonmediated diffusion, facilitated transport involves the reversible binding of the solute to a carrier molecule which transports the solute across the cell membrane. To learn more about this step in the glucose uptake system in brain, a series of experiments were performed to measure the binding of D-glucose to crude membrane fractions prepared from rat

cerebral cortex. Initial experiments were done to develop a valid *in vitro* model of binding.

Crude membrane suspensions were incubated in the presence of D-[^{3}H]glucose or D-[^{3}H]glucose plus an excess of unlabelled D-glucose, and after incubation, membrane suspensions were centrifuged or filtered, and were assayed for radioactivity. As shown in Figures 26 and 29, results from the two methods used to stop the binding reactions did not differ, and the data were pooled. The radioactivity present in each sample was used to calculate the amount of D-[^{3}H]glucose bound and results were usually expressed as pmoles bound per mg of protein in the membrane suspension. Total binding was defined as the radioactivity bound in the absence of unlabelled D-glucose, non-specific binding was defined as the radioactivity bound in the presence of excess unlabelled D-glucose, and the variable of interest, specific D-[^{3}H]glucose binding, was defined as the difference between total binding and non-specific binding. Estimated from Figure 26, 3.7 to 4.7 pmoles of ligand were bound per mg protein in the membrane suspension in the presence of 14.5 to 15.2 nM D-[^{3}H]glucose. Bound radioactivity was displaced by increasing concentrations of unlabelled D-glucose, and displacement was maximal with 50 µM unlabelled D-glucose. Bound radioactivity was not further displaced by unlabelled D-glucose at concentrations up to 300 µM. This non-displaceable radioactivity was called non-specific binding. Radioactivity bound to membrane suspensions which were denatured by heat was all non-specific. As a result of this study, 50 µM was the concentration of unlabelled D-glucose chosen to displace D-[^{3}H]glucose bound in the non-specific binding samples.

Because binding kinetics are measured when the reaction is at

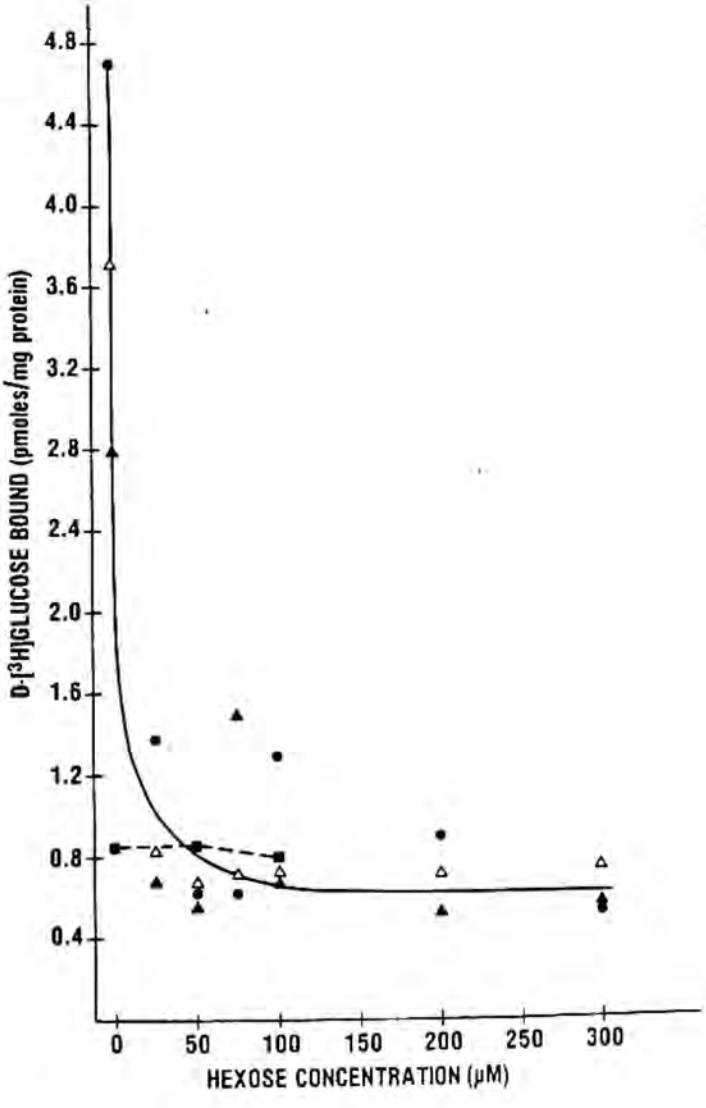

Fig. 26. D-[^{3}H]Glucose binding to synaptic membranes and its displacement by excess D-glucose. Membranes were incubated with 14.5 (Δ,▪) or 15.2 nM (●,▲) D-[^{3}H]glucose in the presence of the indicated concentrations of unlabelled glucose. Heat denatured membranes (▪) were prepared by boiling for 15 min. Binding was stopped by centrifugation (Δ,●,▪) or by filtration (▲). Each point is the mean of three or four incubations, and different symbols represent separate experiments. (Modified from Gold and Kyle-Lillegard, 1983).

equilibrium, D-[^{3}H]glucose binding was studied as a function of incubation time to determine the time necessary for the binding to reach equilibrium. Results are shown in Figure 27. Specific D-[^{3}H]glucose binding appeared to reach equilibrium in 15 min and remained at equilibrium for at least 28 min. Non-specific binding was maximal at 5 min and did not change during the incubation periods studied. From the results of this study, a 15 min incubation time period was chosen for binding studies because at this time period binding was in apparent equilibrium.

An important consideration in binding studies is the demonstration of a linear relationship between binding and tissue concentration. A non-linear relationship might indicate the presence of an endogenous ligand or degradation of the added ligand or of the receptor (Burt, 1978). To test whether D-[^{3}H]glucose binding was a linear function of crude membrane protein concentration, the effect of increasing protein concentration on the radioactivity bound to membrane fractions was studied. As shown in Figure 28, specifically bound D-[^{3}H]glucose increased as protein concentration increased, and the relationship between binding and protein concentration was linear between 0.044 and 0.672 mg protein per sample. In most of my binding studies, the protein concentration was between 0.15 and 0.4 mg per sample.

Binding was studied at steady state to determine the affinity of the binding site in crude membranes for D-[^{3}H]glucose, and results are presented in Figure 29. D-[^{3}H]Glucose binding to crude membrane fractions was studied as a function of D-[^{3}H]glucose concentration. The Inset shows that specific binding appeared to saturate, while non-specific binding did not appear to saturate. The Main Figure shows a Scatchard plot of specific binding from the Inset. The ratio of D-[^{3}H]glucose bound (B) to

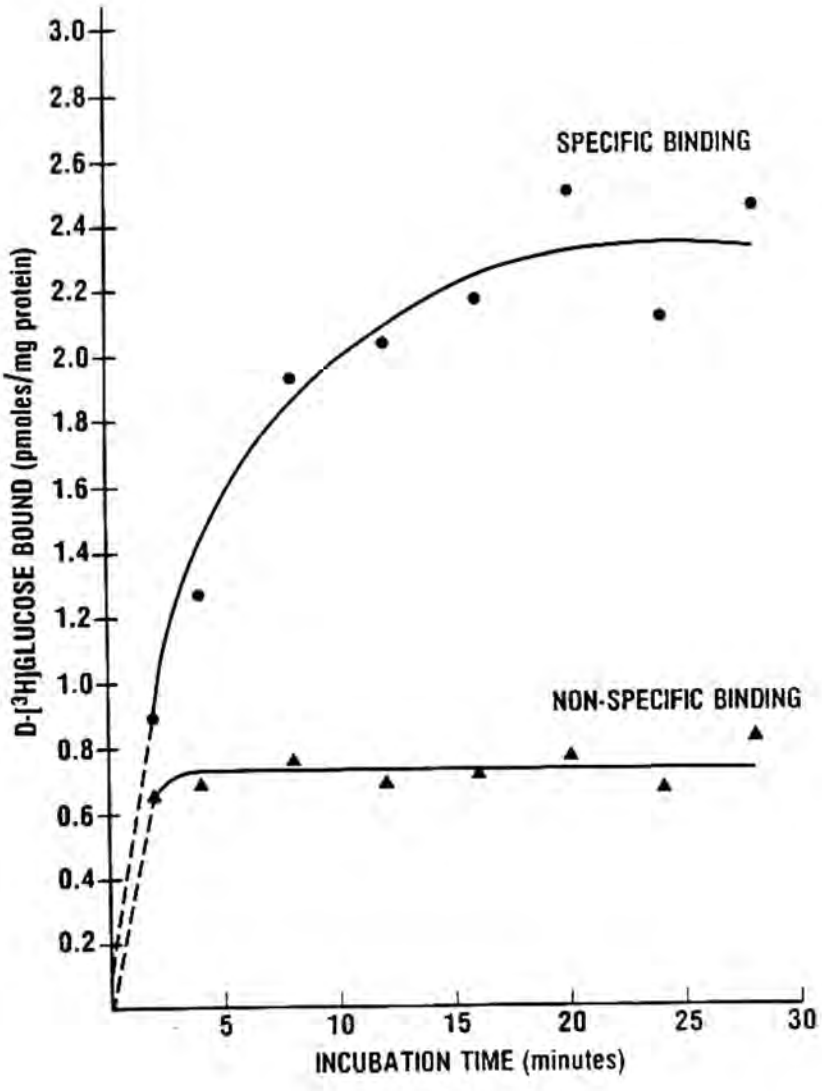

Fig. 27. D-[^{3}H]Glucose binding to synaptic membranes as a function of incubation time. Non-specific binding is radioactivity bound to the membranes in the presence of 50 μM D-glucose. Membranes were incubated with 15.2 nM D-[^{3}H]glucose. Binding was stopped by filtration. Each point is the mean of two to five samples. (Modified from Gold and Kyle-Lillegard, 1983).

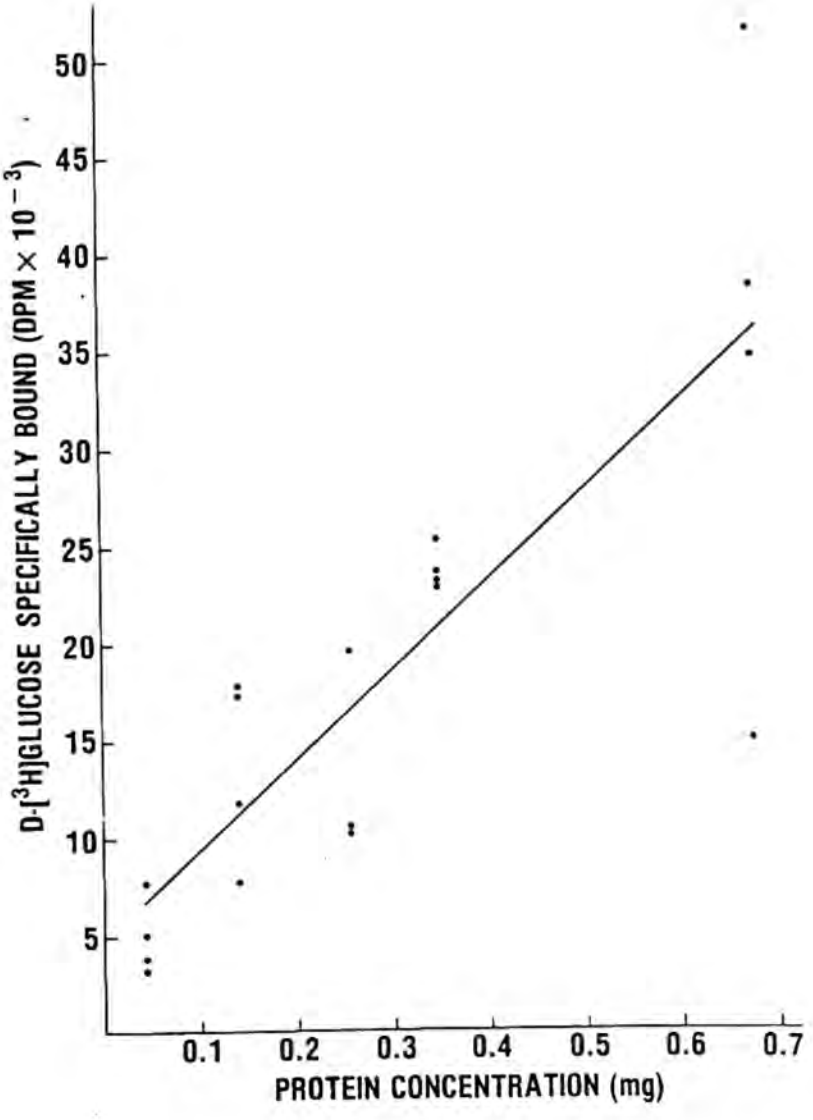

Fig. 28. D-[^{3}H]Glucose binding to crude membrane fractions as a function of protein concentration. Membranes were incubated with 15.2 nM D-[^{3}H]glucose. Binding was stopped by filtration. Each point represents a single sample. The line was estimated by linear regression analysis.

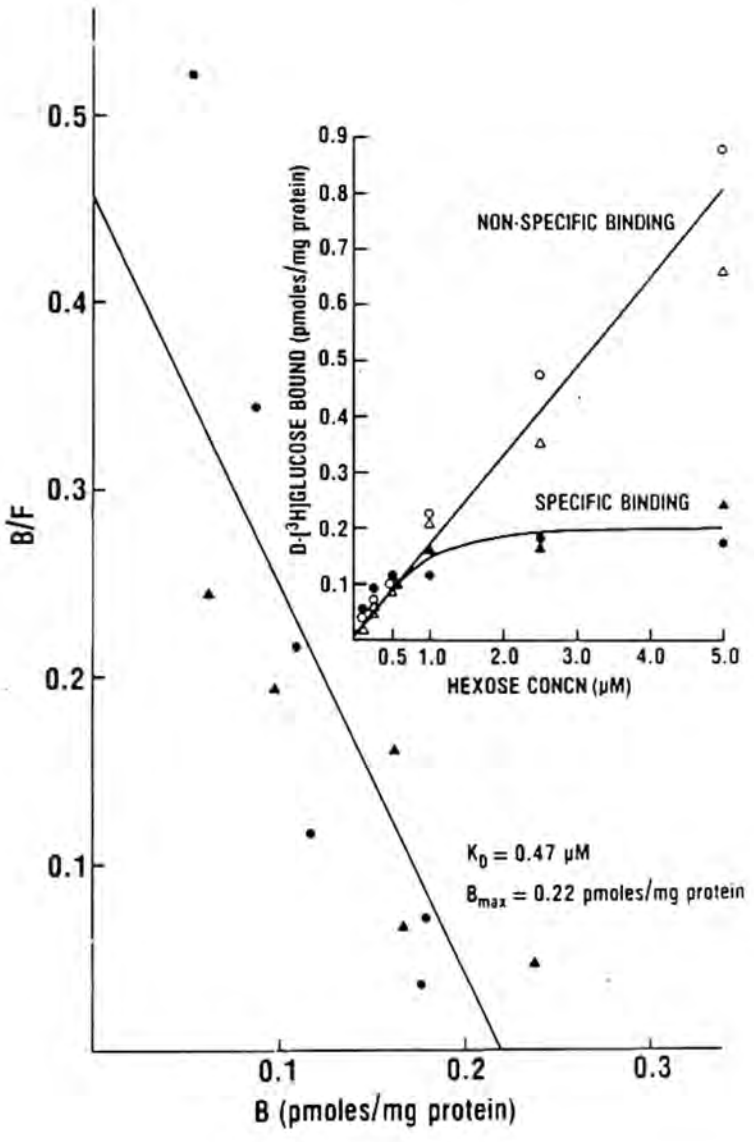

Fig. 29. Inset--Specific and non-specific D-[^{3}H]glucose binding to synaptic membranes. Non-specific binding is radioactivity bound to the membranes in the presence of 50 μM D-glucose. Membranes were incubated in the presence of 14.5 nM D-[^{3}H]glucose, and binding was stopped by centrifugation (●,○); or membranes were incubated in the presence of 15.2 nM D-[^{3}H]glucose and binding was stopped by filtration (▲, Δ). Each point is the mean of two to four incubations, and different symbols represent separate experiments. Main Figure--A Scatchard plot of specific binding displayed in the Inset. (Modified from Gold and Kyle-Lillegard, 1983).

the concentration of D-[^{3}H]glucose present in the medium (F) was plotted as a function of the D-[^{3}H]glucose bound. The straight line estimated from all the points shown yielded the equilibrium binding constant, K_D, and the maximum number of binding sites, B_{max}. The K_D = 0.47 μM is defined as the negative reciprocal of the slope of the regression line. B_{max} is the intercept on the abcissa, estimated to be 0.22 pmoles/mg protein. If only the data from the filtration experiment were used to calculate the equilibrium constants, K_D = 0.88 μM and B_{max} = 0.27 pmoles/mg protein would be obtained. Calculation of these constants using binding data from the centrifugation experiment only yielded K_D = 0.29 μM and B_{max} = 0.18 pmoles/mg protein. Regardless of the points used to estimate these constants, they are very low compared to the kinetic constants obtained from [^{3}H]2DG uptake studies.

Because the equilibrium binding constant K_D is defined as the ratio of the rate of dissociation to the rate of association (Bennett, 1978), it was important to see whether the ratio of the measured rates of dissociation and association yielded results in accord with the K_D estimated from the Scatchard plot. The time-related association and dissociation of D-[^{3}H]glucose are shown in Figure 30, Panel A and B. Data from this study were replotted as described by Bylund (1980) to obtain rate constants for association and dissociation, and these replots are shown in Figure 31, Panels A and B. Dissociation data (Panel B) were replotted as ln (B/B_o) vs time, where B is the amount bound at any time, and B_o is the amount bound at time zero, estimated for this experiment to be 3.04 pmoles/mg protein. The slope of the line generated from plotting ln (B/B_o) vs time is equal to the dissociation rate constant, k_{-1}, and was calculated to be 0.086 min^{-1} for D-[^{3}H]glucose. Data in Panel A were

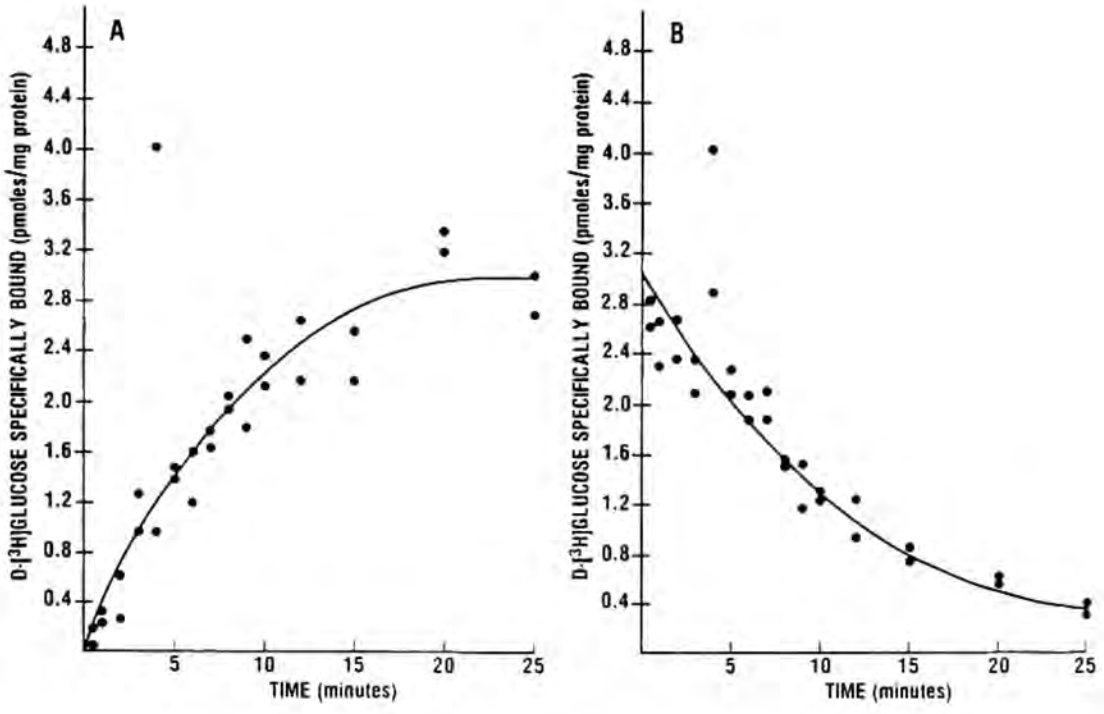

Fig. 30. Panel A--D-[^{3}H]Glucose binding to synaptic membranes as a function of time. B_e was estimated from this plot. Membranes were incubated in the presence of 15.2 nM D-[^{3}H]glucose and the incubation was stopped by filtration. Each point represents a single sample. Panel B--D-[^{3}H]Glucose dissociation from synaptic membranes as a function of time in the presence of 50 μM D-glucose. B_o was estimated from this plot. Incubations were stopped by filtration. Each point represents a single sample. (Modified from Gold and Kyle-Lillegard, 1983).

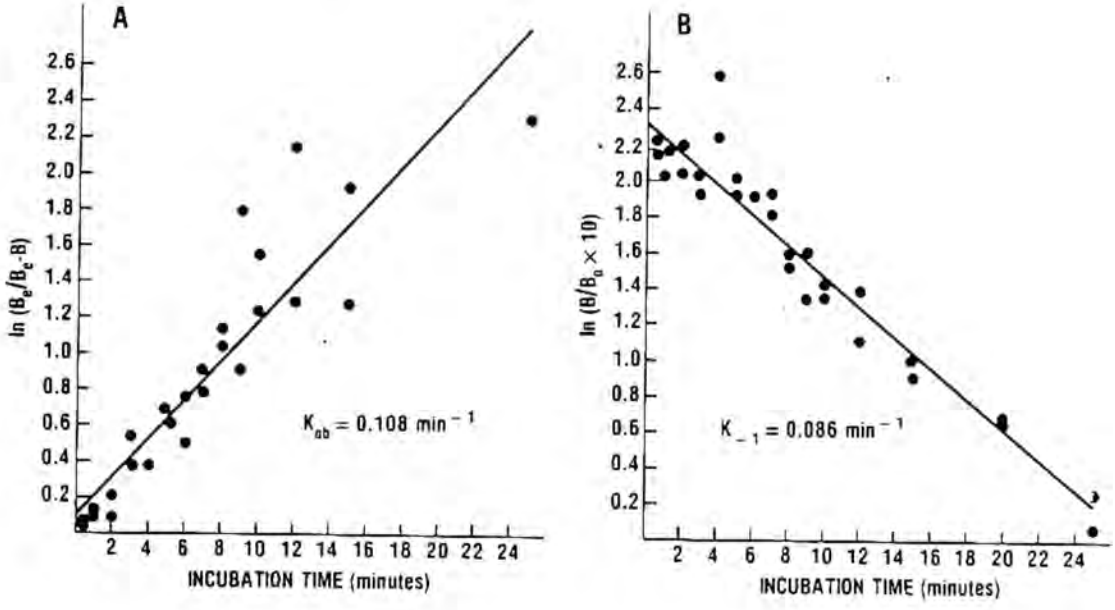

Fig. 31. Replots of association and dissociation data for D-[^{3}H]glucose binding to crude membrane fractions. Data in Panel A were plotted to obtain a line with slope = k_{ob} = 0.108 min^{-1}. Data in Panel B were plotted to obtain a line with slope = k_{-1} = 0.086 min^{-1}.

replotted as ln (B_e/B_e-B) vs time, where B_e is the amount bound at equilibrium, estimated for this experiment to be 2.98 pmoles/mg protein. The slope of this line was k_{ob}, equal to 0.108 min^{-1}. K_{ob} was substituted in the equation $k_{ob} - k_{-1} = k_{+1}(L_T)$, where L_T equals total ligand concentration, here 15.2 nM, to solve for $k_{+1} = 0.0014\ nM^{-1}\ min^{-1}$. These results indicate that the fractional rate of dissociation was over sixty times the fractional rate of association. Furthermore, the value representing the ratio of the rate of dissociation to the rate of association was about one-eighth the value obtained for the K_D from saturation experiments.

To characterize further the binding site for D-[^{3}H]glucose in crude membrane fractions, the ability of several glucose analogues to specifically displace D-[^{3}H]glucose was tested. Binding of D-[^{3}H]glucose to crude membrane fractions was studied in the presence of selected hexoses, and results are shown in Figure 32. As shown previously, binding was inhibited in the presence of D-glucose; the concentration of D-glucose which caused a 50% inhibition of binding (IC50) was estimated by probit analysis (Goldstein et al., 1974) to be 0.31 µM. D-[^{3}H]glucose binding was also inhibited in the presence of 2-deoxyglucose, with an IC50 = 3.26 µM, estimated by probit analysis, and in the presence of 3-O-methylglucose, with an IC50 = 30 µM, estimated from Figure 32. L-glucose had no effect on D-[^{3}H]glucose binding in concentrations up to 50 µM. This result indicated that D-[^{3}H]glucose binding was stereospecific. Binding of D-[^{3}H]-glucose was inhibited by hexoses in a rank order similar to the rank order in which hexoses inhibited [^{3}H]2DG uptake by brain slices.

The membrane preparation routinely used to study D-[^{3}H]glucose binding was prepared as described by Enna and Snyder (1975), and was

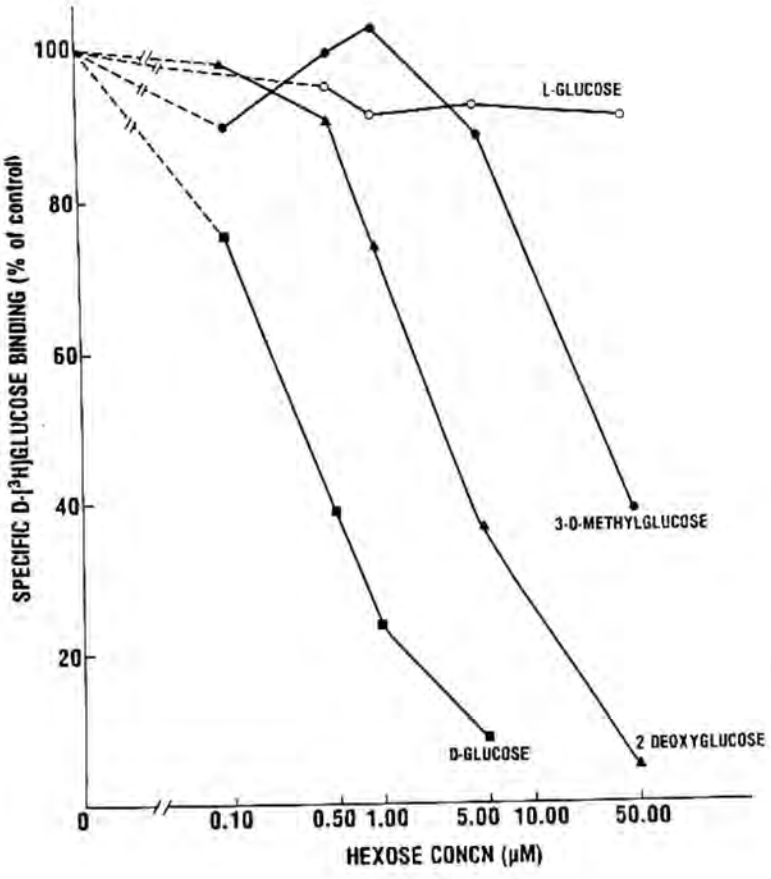

Fig. 32. Displacement by different hexoses of D-[^{3}H]glucose bound to synaptic membranes. Samples were incubated with 14.5 or 15.2 nM D-[^{3}H]glucose in the presence of the indicated concentrations of unlabelled hexoses and data are pooled from several experiments. Binding was stopped by centrifugation (all hexoses except L-glucose) or by filtration (L-glucose). One hundred percent (control), determined for each experiment, was the value obtained for specific binding in the absence of added hexose. Each point is the mean of triplicate samples. (From Gold and Kyle-Lillegard, 1983).

known to be a relatively crude preparation. To test whether D-[^{3}H]glucose binding was to neuronal membranes, I studied the binding of D-[^{3}H]-glucose to membrane preparations purified as described by Gray and Whittaker (1962) and Gurd et al. (1974). I also studied the association of D-[^{3}H]glucose with subcellular fractions enriched in mitochondria. Results of these binding studies are shown in Figure 33. D-[^{3}H]glucose bound to all fractions studied; specific binding was present in a fraction enriched in synaptic membranes ("P_2B"), and a highly purified fraction enriched in synaptic membranes (PSYN). Specific binding was also present in a fraction enriched in mitochondria ("P_2C"), and in a highly purified fraction, resulting from the preparation of the purified synaptic membrane-enriched fraction, known to contain mitochondria (PSYN·MIT).

I had demonstrated the existence of a binding site for D-[^{3}H]glucose in crude membrane fractions prepared from rat brain. D-[^{3}H]glucose binding to this site was displaceable, saturable, and stereospecific. Binding was inhibited by other hexoses in a rank order similar to that for [^{3}H]2DG uptake by brain slices. These findings began to characterize a recognition site for D-glucose in brain, but did not reveal information as to the function of this site. Of many possible functions for this recognition site, two were considered: 1) the site represented the carrier molecule for glucose transport, or 2) the site represented hexokinase, the enzyme which catalyzes the phosphorylation of glucose to glucose-6-phosphate. Some initial studies were done to test whether one or the other of these possible functions for D-[^{3}H]glucose recognition by crude membrane fractions was operating.

The objective of the first study was to determine the effect of selected concentrations of glucose-6-phosphate on D-[^{3}H]glucose binding.

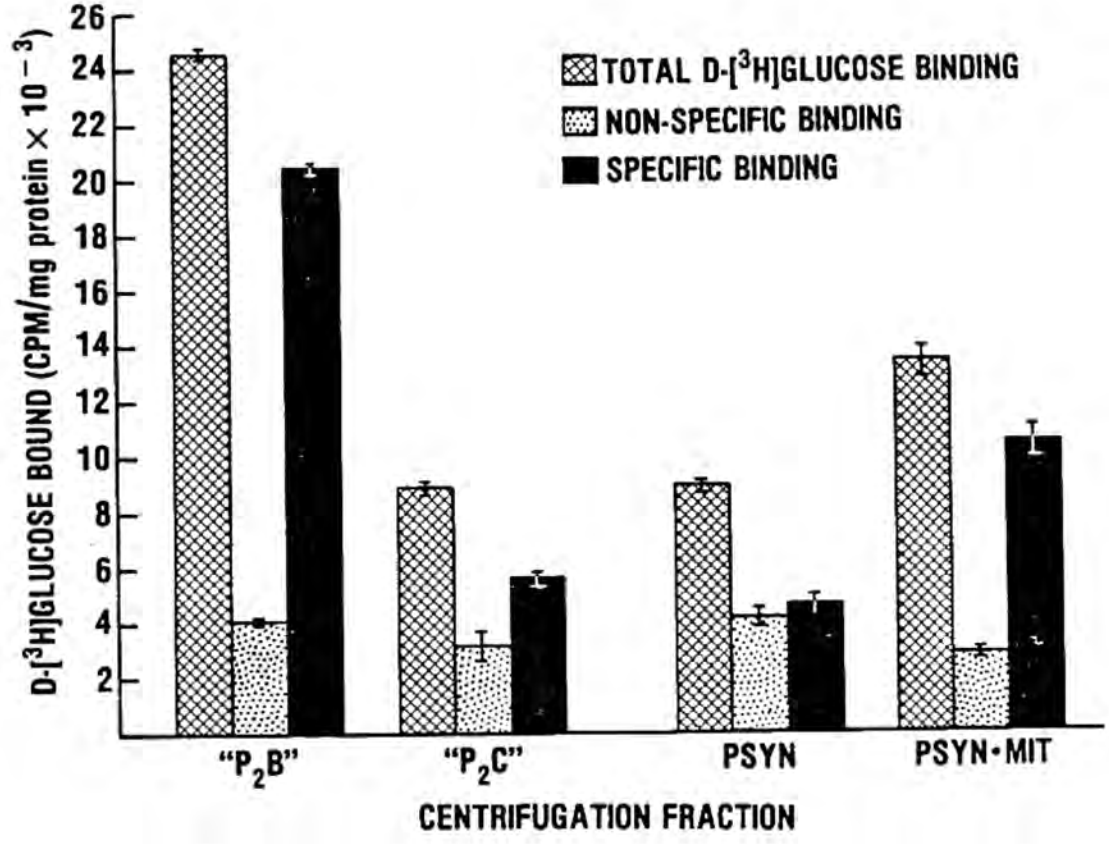

Fig. 33. D-[^{3}H]Glucose binding to different centrifugation fractions. Membranes were incubated with 14.5 nM D-[^{3}H]glucose. Binding was stopped by centrifugation. Preparations are described in the text. The membrane fractions were enriched as follows: "P_2B": synaptosomal membranes, "P_2C": mitochondrial membranes, PSYN: synaptosomal membranes, PSYN•MIT: mitochondrial membranes. Each value is the mean ± SEM of three samples.

Glucose-6-phosphate is a known inhibitor of hexokinase; the K_i for hexokinase is 0.4 mM (Bachelard, 1980). Another effect of glucose-6-phosphate on hexokinase concerns the subcellular distribution of the enzyme. Brain hexokinase has been shown to occur in a soluble form located in the cytosol, and in a particulate form primarily associated with the mitochondria (Bachelard, 1967). Glucose-6-phosphate, in concentrations of approximately 0.1 to 1.0 mM, has been shown to release mitochondrial-bound hexokinase (Wilson, 1968). If the binding reaction I had observed was glucose binding to particulate hexokinase, and added glucose-6-phosphate was causing release of bound hexokinase from the mitochondria, then the result would be a decrease in observed binding. The effect of increasing concentrations of glucose-6-phosphate (G-6-P) on D-[^{3}H]glucose binding to membranes is shown in Figure 34. Binding was inhibited in the presence of G-6-P, and the concentration of this compound which caused a 50% inhibition of specific binding was estimated to be 1.9 μM. This concentration of G-6-P is much lower than the reported K_i for hexokinase or the concentration of G-6-P necessary to solubilize 50% of the bound enzyme (both > 30 μM).

Adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) also have the ability to release to the cytosol hexokinase from the mitochondria (Wilson, 1968). ATP is a cosubstrate for D-glucose phosphorylation by hexokinase, and ADP is a competitive inhibitor of this reaction (Sols and Crane, 1954). To test whether these compounds had an effect on the observed binding of D-[^{3}H]glucose binding to crude membrane fractions, the effect of ADP and ATP on the binding of D-[^{3}H]glucose to a fraction enriched in synaptic membranes ("P_2B") was studied, and data are presented in Figure 35. Specific binding was not changed when 50 μM L-glucose was present, as already shown (Figure 32). Specific binding

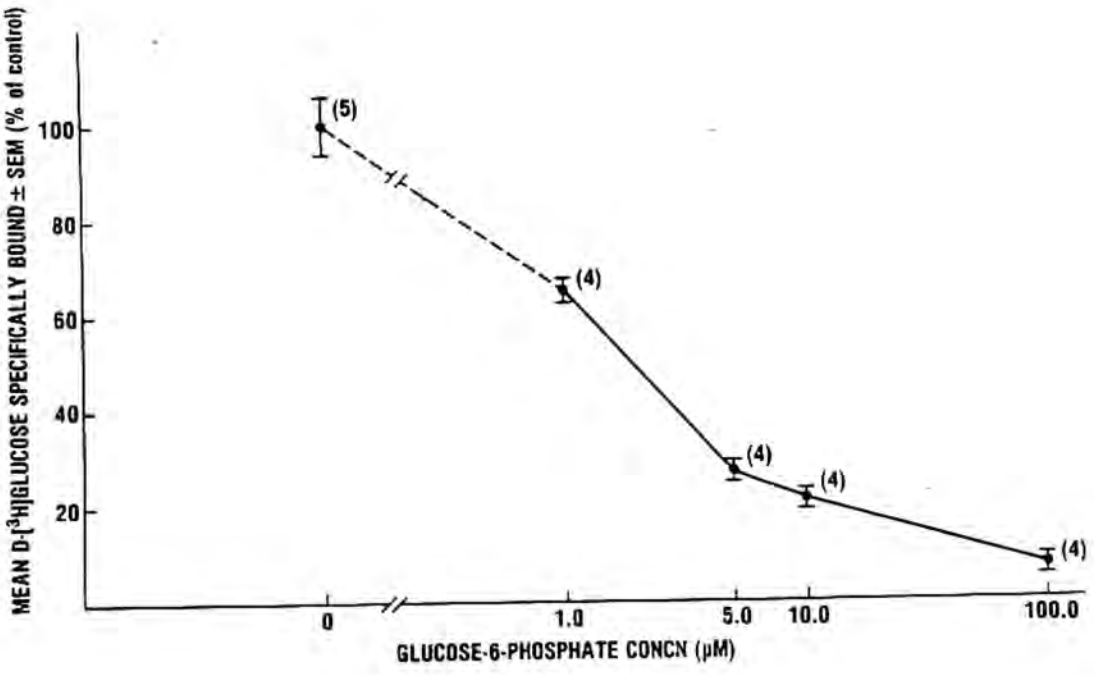

Fig. 34. Displacement by glucose-6-phosphate of D-[^{3}H]glucose bound to synaptic membranes. Samples were incubated with 15.2 nM D-[^{3}H]glucose in the presence of the indicated concentrations of glucose-6-phosphate. Binding was stopped by filtration. Each value is the mean $\pm$ SEM of the number of samples shown.

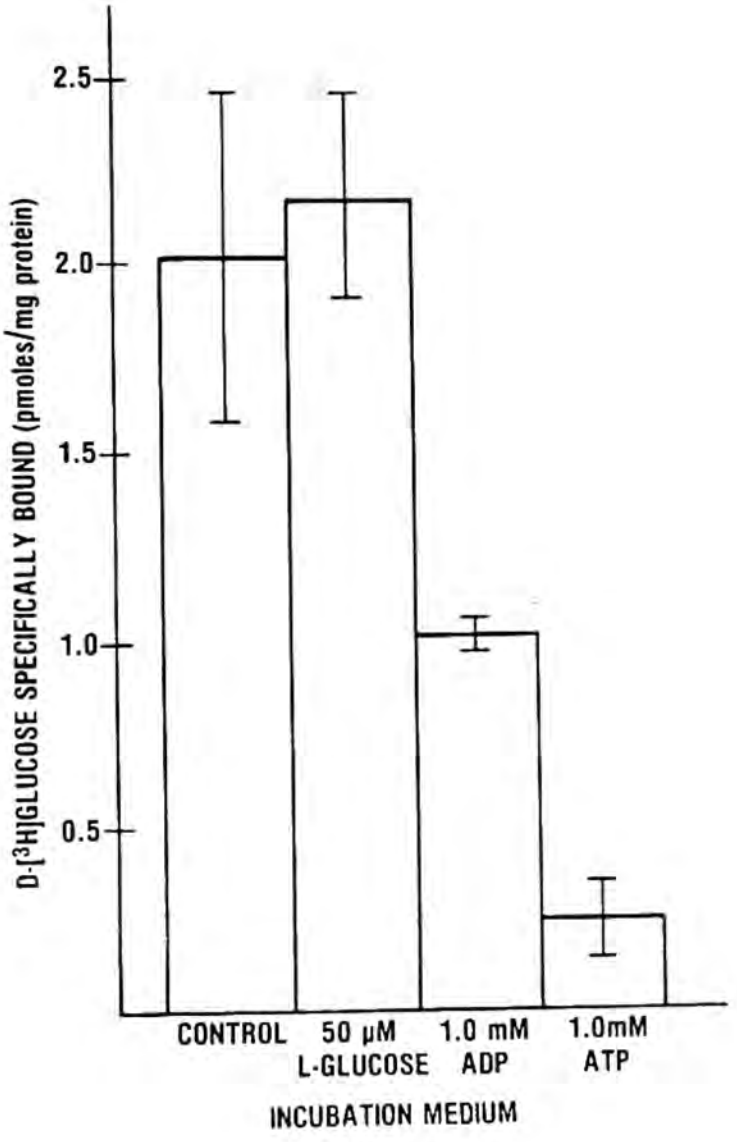

Fig. 35. D-[^{3}H]Glucose binding to P_2B membrane fractions: effect of ADP and ATP. Membrane fractions were incubated with 14.5 nM D-[^{3}H]glucose in the presence of 50 µM L-glucose, or 1.0 mM ADP, or 1.0 mM ATP. Binding was stopped by centrifugation. Values are means ± SEM of triplicate incubations.

was reduced by about 50% in the presence of 1.0 mM ADP, and it was reduced by about 85% in the presence of 1.0 mM ATP. I tested the possibility that D-[^{3}H]glucose was being metabolized in the presence of ATP by applying a fraction of the supernatant obtained from centrifugation of the samples at the end of the binding experiment to an ion exchange column. Over 90%, and in most cases, 99% of the radioactivity recovered coeluted with neutral glucose indicating that there was no significant metabolism of D-[^{3}H]glucose under these conditions. This reduction of binding in the presence of ADP and by ATP suggests, but does not confirm, that the binding reaction observed is to hexokinase.

In summary, these data demonstrate the existence of a binding site for D-[^{3}H]glucose in a crude membrane fraction prepared from rat cerebral cortex. D-[^{3}H]glucose binding to this fraction was displaceable, stereospecific, saturable, and of a very high affinity (K_D = 0.47 μM). Binding was inhibited by other hexoses known to be transported by the D-glucose carrier. Specific binding was observed in crude membrane fractions as well as in highly purified synaptic membrane-enriched fractions, and also in mitochondrial enriched fractions. The function of this binding site has not been determined from these studies, and while the possibility remains that this binding represents binding to a carrier for D-glucose transport, there is other evidence to suggest that this binding is to hexokinase.

DISCUSSION

Uptake Studies

Glucose is essential for normal cerebral metabolism and neuronal function in most circumstances. Many *in vitro* studies of glucose transport by brain have been done during the past fifteen years using various tissue preparations and hexose analogues. In general, brain cell hexose transport was found to be carrier-mediated (Bachelard, 1971; Diamond and Fishman, 1973), sensitive to inhibition by phloretin (Diamond and Fishman, 1973; Walum and Edström, 1976a; Halton et al., 1980), and sodium independent (Diamond and Fishman, 1973; Lund-Andersen et al., 1976; Halton et al., 1980). Much of this information was obtained with the synaptosome and cultured cell preparations. There is a paucity of data from hexose uptake studies using the brain slice preparation. Early work with slices were not informative because high sugar concentrations were used, and kinetic constants determined for hexose uptake were large. More recent studies using this tissue preparation revealed that hexose uptake by slices was kinetically similar to hexose uptake by synaptosomes. I have extended the studies of hexose transport by brain slices, using the uptake of [^{3}H]2-deoxyglucose ([^{3}H]2DG) to model glucose uptake. I adapted the use of L-[^{3}H]glucose as a control for space available for glucose diffusion. In addition, I have begun to study possible regulatory influences on the [^{3}H]2DG uptake model. These data both support and further extend the existing data for glucose transport by brain *in vitro*.

As noted above, glucose transport across brain cell membranes was modelled *in vitro* by the uptake of radiolabelled 2-deoxyglucose by rat cerebral cortex slices. The brain slice preparation, originally introduced by Warburg in the 1930's (see Elliott, 1969), has been used for metabolic studies (McIlwain et al., 1951; Machiyama et al., 1970), studies of amino acid uptake (Banay-Schwartz et al., 1971), and studies of glucose uptake (Bachelard, 1971). Brain slices contain intact cells that maintain resting membrane potentials. They have a full complement of metabolic enzymes, and increase their glycolytic rates as a result of electrical stimulation. One particular advantage of the use of this tissue model is that much of the original cytoarchitecture is intact (Bachelard, 1976). Disadvantages include some structural damage due to cut edges; these damages could cause release of intracellular enzymes to the surrounding medium. Also, there is a large space available for diffusion which is often increased by damage and swelling (Pappius, 1969). Brain slices contain both neurons and glia, but they do not rely on the vascular system for supply of nutrients (Bachelard, 1975).

Because brain slices contain a large space available for diffusion, it is important to include a suitable control in the experimental design to measure this space. Many different compounds have been used to approximate the extracellular space in brain slices; these include inulin, sucrose, mannitol, dextran, and raffinose. Pappius (1969), in a review of fluid spaces in whole brain, concluded that the extracellular space was 15 to 25% of total brain volume. I used L-glucose to estimate the space available for glucose diffusion. L-glucose is structurally identical to D-glucose, and therefore produces the same tonicity in the medium, and similarly affects tissue swelling or shrinkage. L-glucose

is not transported into the cell by a carrier system because carrier systems are generally stereoselective (Lehninger, 1975). For these reasons, L-glucose is a valid control for estimating the space available for glucose diffusion. L-glucose distributed to approximately 80 to 90% of the slice wet tissue weight, and therefore measured both extracellular and intracellular space. Others have reported the similar use of radiolabelled L-glucose to determine space available for diffusion in synaptosomes (Heaton and Bachelard, 1973; Tan et al., 1977).

The uptake of [^{3}H]2DG was used to model the uptake of D-glucose. Of the many glucose analogues available for studies of cerebral glucose uptake, 2DG is advantageous to use because of its metabolic properties. It is taken up by brain tissue in a manner similar to D-glucose. After entering the tissue, 2DG competes with endogenous D-glucose for phosphorylation by hexokinase. 2-Deoxyglucose-6-phosphate is not further metabolized by the cell; it is not a substrate for phosphohexoseisomerase, and phosphatase activity in brain is low (see Sokoloff et al., 1977). As a result, 2DG-6-phosphate is trapped in the cell, and the amount of this metabolite can be measured. Although the phosphorylation of 2DG is an advantage in that the product is trapped in the cell, it is a disadvantage in that the uptake process and the phosphorylation process may not be clearly distinguished from each other. The calculation of accumulated hexose and hexose phosphate may then be a reflection of the uptake or the phosphorylation, without clear information as to which process is rate-limiting.

Net [^{3}H]2DG uptake was defined as the difference between [^{3}H]2DG uptake and L-[^{3}H]glucose uptake by slices in parallel samples. Under standard conditions, using 0.5 mM hexose, the sample ([^{3}H]2DG uptake) to

blank (L-[^{3}H]glucose uptake) ratio was 2 to 1. This ratio was somewhat lower at higher hexose concentrations (5 mM) where a larger percentage of [^{3}H]2DG uptake was by diffusion. The concentration of hexose used (0.5 mM) was similar to that used by Fletcher and Bachelard (1978) who measured high affinity 2DG uptake by brain slices.

Initial studies of the uptake of radiolabelled 2DG were done to characterize the uptake system in slices. Small changes in osmolarity produced by varying choline chloride or L-glucose concentrations did not change the uptake of [^{3}H]2DG. As neither compound had an effect on net [^{3}H]2DG uptake by slices, they were used interchangeably to control for changes in osmolarity. The lack of effect of L-glucose on [^{3}H]2DG uptake suggests that the uptake of 2DG and the uptake of L-glucose proceed by different mechanisms. This is consistent with the hypothesis that 2DG uptake is carrier mediated and the uptake of L-glucose is by diffusion alone. The increase in L-[^{3}H]glucose uptake seen when the concentration of L-glucose in the incubation medium was increased, was directly proportional to the change in hexose concentration. This observation further supports the hypothesis that L-[^{3}H]glucose uptake is by diffusion.

The uptake system was studied as a function of incubation time, and the initial rate of uptake was determined. Net [^{3}H]2DG uptake was linear for at least 10 min, and a 2 min incubation period was used as an estimate of initial rate in all subsequent studies. L-[^{3}H]glucose uptake appeared to reach equilibrium by 1 min, and it was unchanged during longer incubation times. This satisfies one important criterion of valid fluid space measurements (Pappius, 1982). Similar estimations for initial rates of hexose uptake by brain were described by others (Heaton and

Bachelard, 1973; Diamond and Fishman, 1973; Fletcher and Bachelard, 1978). By comparison, hexose uptake is much more rapid in erythrocytes (Britton, 1957), adipocytes (Gliemann, 1982), and some cultured cells (Plagemann et al. 1981; Walum and Edström, 1976b).

Temperature dependence was a characteristic of net [^{3}H]2DG uptake by brain slices. While the temperature coefficient determined for net uptake in the present studies (Q_{10} = 1.87 from 15° to 25°) was higher than the value usually associated with passive diffusion, it could have represented a complex type of diffusion or a carrier mediated event. Cooke and Robinson (1971) determined a Q_{10} = 1.4 for 3-O-methylglucose uptake by rat brain slices. Diamond and Fishman (1973) reported a somewhat higher Q_{10} for [^{3}H]2DG uptake by synaptosomes. Although the temperature coefficient for net [^{3}H]2DG uptake by cerebral cortex slices determined in the present studies does not define the mechanism of uptake, it suggests that uptake is carrier mediated, which is consistent with the conclusions of earlier workers.

Cotransport with sodium is a property of the active transport system for D-glucose by intestine and kidney. Uptake of [^{3}H]2DG by brain slices was not changed when sodium was deleted from the incubation medium. Furthermore, there was no effect on net [^{3}H]2DG uptake when K^+, Ca^{2+}, Mg^{2+}, or Cl^- were individually deleted. Similar results were shown by Diamond and Fishman (1973) who studied [^{3}H]2DG uptake by synaptosomes; but these authors did see a small effect of sodium on uptake. Wheeler and Hollingsworth (1979) demonstrated an inhibitory effect of sodium on 2DG uptake by synaptosomes from the Long-Evans rat. These data suggest that glucose is not cotransported with sodium in the brain.

Whereas studies of brain hexose uptake in vitro show no dependence on sodium, changing concentrations of sodium or other components of the incubation media does affect other processes in slices. For example, the absence of glucose or deletion of sodium has been shown to increase intracellular space in the tissue slice preparation (Harvey and McIlwain, 1969). In my studies, the swelling would have been reflected by changes in the uptake of L-[^{3}H]glucose which were not detected throughout these experiments.

To be certain that the uptake system I was studying with [^{3}H]2DG was specific for D-glucose as well, I tested the ability of D-glucose to inhibit the net uptake of [^{3}H]2DG. D-glucose inhibited net [^{3}H]2DG uptake by brain slices, and the IC50 was estimated to be about 0.6 mM. Net [^{3}H]2DG uptake was also inhibited by unlabelled 2-deoxyglucose; this was expected considering the saturability demonstrated for [^{3}H]2DG uptake. The analogue 3-O-methylglucose also inhibited, although very slightly, the net uptake of [^{3}H]2DG, probably reflecting the high K_i (7 mM) of this compound for [^{3}H]2DG uptake reported by others (Diamond and Fishman, 1973). These competition studies verify that [^{3}H]2DG uptake models physiological hexose transport by brain.

Transport processes have characteristic affinities for their substrates, and maximal capacities for transport. When these characteristics were determined for [^{3}H]2DG uptake, L-[^{3}H]glucose uptake, and the net uptake of [^{3}H]2DG, the uptake of [^{3}H]2DG showed saturability, while L-[^{3}H]-glucose uptake did not. Saturability is a criterion of carrier-mediated transport and non-saturability is a property of diffusion kinetics. This is further evidence that L-[^{3}H]glucose uptake is a diffusion process while uptake of [^{3}H]2DG is carrier-mediated. The K_m = 1.85 mM estimated

for net [^{3}H]2DG uptake is somewhat greater than the K_m determined by others for 2DG uptake into synaptosomes (0.24 to 0.55 mM; Diamond and Fishman, 1973; Heaton and Bachelard, 1973), but it is similar to that determined for 2DG uptake into dense-cultured glioma (5.2 mM; Walum and Edström, 1976b). For comparison, glucose uptake by adipocytes has a K_m of approximately 8 mM (see Gleimann, 1982), and the K_m for hexose uptake by erythrocytes is approximately 6.2 mM (Lehninger, 1975). These results suggest that brain cells have a slightly higher affinity for hexoses when compared to adipocytes or red cells.

The inhibitory actions of the chemically-related compounds phlorizin and phloretin on D-glucose transport by other tissues are well known. That neither compound inhibited net [^{3}H]2DG uptake by cerebral cortex slices suggests that this uptake mechanism differs from the Na^+-independent system described for erythrocytes (sensitive to inhibition by phloretin) as well as from the Na^+-dependent system described for kidney or intestine (sensitive to inhibition by phlorizin). Alternatively, if the uptake system were primarily a measure of phosphorylation (as has been suggested by Lund-Andersen and Kjeldsen, 1977), it would not be sensitive to these inhibitors because they are established as inhibitors of uptake. In contrast to my results, phloretin inhibited the uptake of certain hexoses by synaptosomes (Diamond and Fishman, 1973; Tan et al., 1977; Halton et al., 1980) and by glioma and neuroblastoma cells in culture (Edström et al., 1975; Walum and Edström, 1976a). In agreement with the present studies, Cooke and Robinson (1971) found no effect of phlorizin on 3-O-MG uptake by brain slices. Phlorizin did inhibit [^{3}H]3-O-MG uptake by glioma and neuroblastoma cells in culture (Edström et al., 1975; Walum and Edström, 1976a), but the concentrations used were 50

fold higher than the concentration of phloretin which caused a similar inhibition. Both phlorizin and phloretin have other effects at much higher concentrations than those at which glucose transport is inhibited (Silverman, 1976; Crane, 1960). Because phlorizin and phloretin are, however, specific, and the concentrations tested were at least 10 times the reported K_i's for inhibition of glucose uptake, I conclude that the uptake of [^{3}H]2DG by brain slices is not similar to other known hexose uptake systems with respect to sensitivity to inhibition by phlorizin or phloretin.

To develop further a comparison between characteristics of hexose uptake by brain slices and other tissues, I tested the sensitivity of [^{3}H]2DG uptake to metabolic inhibitors. Energy dependence is a characteristic of the phlorizin sensitive, Na^+-dependent hexose uptake system in kidney and intestine. By contrast, the phloretin sensitive, Na^+-independent hexose uptake system in the red blood cell is energy independent. In the present studies, uptake of [^{3}H]2DG was inhibited in the presence of DNP, but not in the presence of sodium azide. DNP is an uncoupler of oxidative phosphorylation (Slater, 1967), and has been shown to cause increased respiration in brain slices (McIlwain and Gore, 1951). Similar findings of DNP inhibition of [^{3}H]2DG uptake by synaptosomes were reported (Diamond and Fishman, 1973; Wheeler and Hollingsworth, 1979). Sodium azide, an inhibitor of cytochrome oxidase (Doull, 1980), did not inhibit net [^{3}H]2DG uptake by brain slices in the concentrations tested. This result might be explained by an incomplete inhibition of cytochrome oxidase (Slater, 1967). These results suggest that the uptake of [^{3}H]2DG by brain slices is dependent, to a small extent, on metabolic energy. It is also possible that these results were due to decreased hexokinase-

catalyzed phosphorylation, which requires ATP. Inhibition at this step could cause decreased net uptake of [^{3}H]2DG by increasing the relative amount of free intracellular [^{3}H]2DG.

To verify that the DNP effects were on the energy charge of the cells, studies were designed to alter intracellular ATP stores, and test the subsequent ability of slices to take up [^{3}H]2DG. The results from preincubation studies in which slices were exposed to D-glucose or L-glucose with or without DNP, confirmed the results obtained when DNP was present during the incubation period only. Results obtained when slices were incubated with D-glucose were not statistically different compared to results from slices which had not been preincubated. The results with L-glucose were surprising, because net [^{3}H]2DG uptake was unchanged after slices were incubated for up to 1 h in the absence of an energy substrate. This suggested, among other possibilities, that in the absence of a metabolic substrate, the brain slices were inactive during the preincubation period. Their increased ability to take up [^{3}H]2DG after this period when compared to slices which had been preincubated in D-glucose may represent an activation of uptake.

The relationship between slice ATP levels and the ability of the slice to take up [^{3}H]2DG was not straightforward. There was a good correlation between these two variables when the slices were exposed to DNP. However, in all other cases increases or decreases in uptake were not readily explained by parallel changes in ATP levels. Banay-Schwartz et al. (1971; 1974) described similar results for amino acid transport, which requires energy, by mouse brain slices in the presence of various metabolic inhibitors.

Studies were done to clarify further the effect of extended pre-

incubation with or without an energy supplying substrate on the subsequent ability of the slice to take up [^{3}H]2DG. The results were in agreement with the results from the study described above. That uptake was activated after preincubation in the absence of a glycolytic substrate was confirmed in these studies. Furthermore, preincubation in the presence of D-glucose did not activate or otherwise change the uptake system as compared to control. One explanation of these results is that during preincubation in the absence of D-glucose, intracellular stores of D-glucose were depleted. The decreased intracellular glucose availability may have initially activated hexokinase, increasing the rate of the entire glycolytic pathway and the uptake step. There is some support for this in the literature. In hypoglycemia, depletion of glucose stores is followed by decreased neuronal activity and decreased glucose utilization (Gorell et al., 1977). Several investigators measured levels of brain glycolytic intermediates during hypoglycemic insult and recovery in mice or rats (Lewis et al., 1974b; Dirks et al., 1980; Ratcheson et al., 1981). In general, they found that all intermediates were decreased during hypoglycemia. One group (Gorell et al., 1977) reported a small but not statistically significant decrease in the level of glucose-6-phosphate. Because of this observation they suggested that glucose transport was enhanced under these conditions. When glucose is depleted intracellularly, and glucose availability is limited, as in the case of hypoglycemia, transport of glucose becomes rate-limiting for glucose utilization (Bachelard, 1980). The extended preincubation conditions used here may have been an *in vitro* approximation of hypoglycemic events *in vivo*. The ability of D-glucose to immediately reverse hypoglycemic stupor is well known.

It is apparent that activation of [^{3}H]2DG uptake might be a result of either increased transport or increased hexokinase activity. How the activation was triggered was addressed as follows. I expanded the preincubation studies to include the addition of several compounds in the preincubation medium which had some activity in the hexose uptake system. For example, when 3-O-MG was added to the preincubation medium, the subsequent ability of the slices to take up [^{3}H]2DG was enhanced. If the signal for enhanced uptake were decreased activity of a carrier, then 3-O-MG would not initiate the signal because it apparently enters cells by the same system as D-glucose. When pyruvate was added to the preincubation medium, the subsequent ability of the slices to take up [^{3}H]2DG was also enhanced. If the signal for increased uptake were lack of metabolic energy, then preincubation in pyruvate would not have increased uptake. Pyruvate bypasses glycolysis but is an effective energy supply for brain slices (McIlwain, 1975). Finally, preincubation in 2-deoxyglucose did not change the subsequent uptake of [^{3}H]2DG. This glucose analogue is a substrate for hexokinase. In summary, it is possible that lack of substrate for hexokinase is the signal for enhanced uptake, or that phosphorylation inhibits the enhanced uptake resulting from depleted intracellular glucose.

There is support for these conclusions in the cell culture literature. For example, many authors have reported that incubation of cultured cells in the absence of glucose results in a large enhancement of subsequent hexose uptake and metabolism. In cultures of hamster fibroblasts (NIL strain) grown almost to confluency, uptake of radiolabelled galactose or of radiolabelled 3-O-MG was enhanced after a 20 h incubation period in the absence of glucose. In agreement with my results with brain

slices, the presence of 3-O-MG during this incubation had no effect on the enhancement (Ullrey and Kalckar, 1981). In contrast to my findings, these authors also reported that the presence of 2DG during the preincubation also had no effect on the enhancement of uptake studied subsequently. Similar effects were reported by Salter and Cook (1976) with diploid human cells. Earlier studies with chick fibroblasts showed that 2DG did inhibit the enhancement of uptake resulting from glucose deprivation (Martineau et al., 1972; Kletzien and Perdue, 1975). These results are similar to those obtained in the brain slice model during the extended preincubation studies, and together suggest that the brain can respond to decreased glucose availability by activating an uptake system.

The overall rate for a series of reactions will be governed by the rate of the slowest reaction. The [^{3}H]2DG uptake model adapted to model glucose uptake by brain slices does not distinguish between the transport of this hexose analogue and its subsequent phosphorylation. This has caused some disagreement among researchers studying hexose uptake in vitro. For example, Lund-Andersen and Kjeldsen (1977) measured the accumulation of 2DG and 2DG-6-phosphate by brain slices. As they increased the 2DG concentration in the medium from 3 to 12 mM, in the presence of 6 mM D-glucose, they measured a lower percentage of intracellular 2DG-6-phosphate at the higher concentration of 2DG. These authors fitted the uptake data to a four compartment model to obtain rate constants for transport and phosphorylation. They concluded, on the basis of computed rate constants and the observation that measurable amounts of free 2DG were present intracellularly, that the rate of 2DG uptake was faster than the rate of phosphorylation. In similar experiments, Diamond and Fishman (1973) measured the accumulation of 2DG and 2DG-6-

phosphate by synaptosomes. They used 2DG concentrations ranging from 1 μM to 5 mM, and they found that 25% of the accumulated label was in the form of free 2DG. Because the percent phosphorylated was constant over this concentration range, the authors claimed that phosphorylation was not a rate-limiting step under these conditions. Bachelard (1980) suggested that the presence of D-glucose in the studies done by Lund-Andersen and Kjeldsen (1977) may have affected the phosphorylation of 2DG. This suggestion was based on the fact that the affinity of hexokinase for 2DG is lower than the affinity of this enzyme for D-glucose. Furthermore, glucose-6-phosphate is an inhibitor of hexokinase and the presence of this metabolite may have been responsible for a decreased rate of 2DG phosphorylation.

Because there is disagreement as to whether uptake or phosphorylation is rate limiting for glucose utilization, I estimated the phosphorylation of 2DG in the conditions imposed by my slice model. I first determined the relative amounts of free [^{3}H]2DG and phosphorylated [^{3}H]2DG which accumulated in the slice as a function of incubation time. In agreement with both studies quoted above (Diamond and Fishman, 1973; Lund-Andersen and Kjeldsen, 1977), I was able to detect free intracellular [^{3}H]2DG. The data did not establish a rate-limiting event.

It was possible, however, to use the measure of accumulated phosphorylated hexose to compare changes in the uptake process with changes in the phosphorylation process under different experimental conditions. Such a comparison would reveal if changes in the two processes were correlated. This approach was used to determine if the uptake of [^{3}H]2DG or its subsequent phosphorylation was responsible for the apparent activation of uptake in the extended preincubation studies. Both processes

were found to increase after preincubation in the absence of D-glucose, so the enhancement of uptake observed was either a result of or a stimulus for increased phosphorylation activity.

The uptake of [^{3}H]2DG and the percent phosphorylated [^{3}H]2DG which accumulated after stimulation of the slice by increasing the concentration of potassium ion in the medium were compared. The increased accumulation of [^{14}C]2DG-6-phosphate as a function of neuronal activity has been shown by Sokoloff (Sokoloff et al., 1977), and forms the basis of the [^{14}C]2DG autoradiography metabolic mapping technique. The observation that brain slices increase their metabolic activity with electrical stimulation is well known (McIlwain, 1951). In my studies, the small but significant increase in net uptake of [^{3}H]2DG was accompanied by an almost 2-fold increase in percent phosphorylated [^{3}H]2DG accumulated. From these results, a large increase in hexokinase activity apparently caused an increase in hexose uptake.

In summary, the uptake of [^{3}H]2DG by brain slices was used to model D-glucose uptake by brain cells. Brain slices accumulated [^{3}H]2DG in a stereospecific, saturable manner, consistent with the hypothesis that glucose uptake by brain is carrier-mediated. Net uptake of [^{3}H]2DG was inhibited by other hexose analogues, and the K_m of the uptake system for [^{3}H]2DG was 1.85 mM. Characteristics of the uptake system in brain slices were determined and then compared to the known characteristics of other glucose transport systems. Net uptake of [^{3}H]2DG by brain slices resembled the process in erythrocytes in that it was not dependent on sodium; and it resembled the process in kidney and gut in that it was dependent, although to a small extent, on metabolic energy. However, brain slice [^{3}H]2DG uptake did not resemble either system with respect

to sensitivity to inhibition by phloretin or phlorizin. The ability of brain slices to take up [^{3}H]2DG was not directly correlated with slice ATP levels, and uptake was activated after preincubation in the absence of glucose. Because the transport and subsequent phosphorylation of [^{3}H]2DG were not clearly separated in this uptake model, the phosphorylation reaction was studied. Preincubation of slices without D-glucose and preincubation of slices in the presence of a high external concentration of potassium, caused an increase in the uptake of [^{3}H]2DG as well as an increase in the percent [^{3}H]2DG phosphorylated. These results suggest that the uptake process is regulated by glycolytic activity, or alternatively, the uptake may influence subsequent phosphorylation.

Binding Studies

As mentioned above, the model of [^{3}H]2DG uptake by slices measures the accumulation of both hexose and hexose phosphate, and does not distinguish between transport and phosphorylation under the experimental conditions. A different approach to study this carrier-mediated uptake system was to study the primary event of glucose uptake by brain, the association of glucose with a carrier molecule. To study the association of glucose with its carrier in brain, a simple *in vitro* binding assay was developed.

In a review of receptor binding techniques, Bennett (1978) described several experimental conditions which must be satisfied for the development of any particular binding assay. These conditions included the appropriate choice of a specific radiolabelled ligand, and the use of a suitable blank. The ligand chosen for the present studies of D-glucose binding to crude membrane fractions was D-[^{3}H]glucose. I was able

to use authentic D-[^{3}H]glucose in these binding studies and not a glucose analogue because I showed that under the experimental conditions employed there was no appreciable metabolism of glucose. The compound chosen as a blank was unlabelled D-glucose, suitable because of its identity with the radiolabelled ligand.

The binding assay involved the incubation of crude membrane suspensions in the presence of D-[^{3}H]glucose (total binding), or in the presence of D-[^{3}H]glucose plus an excess of unlabelled D-glucose (non-specific binding). The assay was terminated by centrifugation or by filtration to separate the associated ligand from the unbound ligand. Both of these methods were found to be suitable for the studies presented here. While both methods gave similar results, the filtration method was preferred because it allowed for a larger capacity assay.

The membranes used for most of these studies were prepared as described by Enna and Snyder (1975). This membrane preparation results in the isolation of a fraction enriched in synaptic membranes. While this fraction is undoubtedly contaminated to some extent by other intracellular constituents, e.g. glia, ribosomes, it has been used for studies of neurotransmitter binding (Enna, 1978). To be sure specific binding of D-[^{3}H]glucose was to neuronal membranes and not to one of these contaminants an enriched membrane fraction was prepared by combining the methods described by Gray and Whittaker (1962) and Gurd (Gurd et al., 1974). Specific binding was observed in all fractions tested, including two mitochondrial fractions. The amount of non-specific binding, expressed per mg protein, was similar in each membrane fraction tested. Total binding, and therefore specific binding, was highest in the lysed synaptosomal ("P_2B") fraction. If D-[^{3}H]glucose were binding to a synaptic

membrane component, one would expect the specific binding to be enhanced with further purification of the membrane. This was not the case in these studies, where specific binding to the purified preparation (PSYN) was much less than that to P_2B. One interpretation of this result may be that during the purification process, the ability to bind glucose was partially destroyed. Another explanation for the observed results is that the preparations were not as pure as in the published method. Furthermore, although the assay conditions were optimized for the crude preparation, they may be less than optimal for the different fractions.

Binding affinity constants were measured at equilibrium, and the time for binding to reach equilibrium was estimated to be 15 min. The dissociation equilibrium binding constants were estimated to be K_D = 0.47 μM, and B_{max} = 0.22 pmoles/mg protein. These constants do not resemble the kinetic constants obtained for uptake studies of [^{3}H]2DG by brain slices. They are similar, however, to the binding constants reported for D-glucose binding to other tissue preparations. For example, Eichholz et al. (1969) found K_m = 2 μM for specific D-glucose binding to intestinal brush border membranes prepared from the hamster. Kahlenberg et al. (1971) estimated an apparent K_D of 29 μM to 46 μM at 0° for binding of D-glucose to human red blood cell membranes. Chesney and coworkers (1973) described a high and a low affinity system for D-glucose binding to luminal membranes prepared from rabbit renal proximal tubules; the estimated K_m for the high affinity system was 0.67 μM. Two of these groups (Eichholz et al., 1969; Chesney et al., 1973) estimated K_m from Lineweaver-Burk transformations of initial rate studies, not during equilibrium conditions. In all cases, the binding constants obtained are vastly different from the "corresponding" transport constants. Eichholz

summarized his findings as attributable to some other physiological event rather that the first step in active transport. He based this conclusion on a number of inconsistencies between known properties of the uptake system and the observed properties of binding (Eichholz et al., 1969). Kahlenberg concluded that the binding of D-[^{3}H]glucose to human erythrocyte membranes represented the first step of glucose transport (Kahlenberg et al., 1971). The results obtained by Chesney with the intestinal brush border membrane preparation were discussed in favor of D-glucose binding to a site involved in the transport system for the hexose (Chesney et al., 1973).

The K_D is a ratio of the dissociation rate and the association rate. To test for internal consistency, the dissociation and association rates for D-[^{3}H]glucose binding were determined and their ratio was compared to the value for K_D estimated from the Scatchard analysis. The K_D (0.47 μM) estimated from the Scatchard plot was not the same as k_{-1}/k_{+1} (0.06 μM) estimated from association dissociation studies. One possibility for this inconsistency is that the amount of specific binding was underestimated. This could have resulted from the separation methods used to terminate the binding reaction. If these methods were not fast enough compared to the rate of binding dissociation, then specific binding would be underestimated (Bennett, 1978). If specific binding is underestimated, the K_D estimated from the Scatchard plot would be overestimated, and the B_{max} would be underestimated (Bonifacino and Paladini, 1981). Correction of this inconsistency would require development of a new method for the rapid termination of the binding reaction, or a modification of the present ones.

While the affinity constants revealed the presence of a high-

affinity recognition site for D-glucose, the role of this binding site was not known. One possibility was that glucose was binding to a carrier molecule. The ability of several hexoses to inhibit specific D-[^{3}H]glucose cose binding supported this role for the binding site. Binding was selectively inhibited (D-glucose > 2DG > 3-O-MG), and this selectivity was similar to that for inhibition of [^{3}H]2DG uptake by brain slices. Binding was also stereospecific, as it was not affected by L-glucose. Although stereospecificity and selectivity are important properties of the uptake system, the large difference in kinetic constants for [^{3}H]2DG uptake and D-[^{3}H]glucose binding make it difficult to assign unequivocally a carrier role to the binding site.

Another possibility is that the binding site is the active site of hexokinase. This possibility is supported by the stereospecificity and selectivity of binding for D-glucose and 2DG. Conversely, inhibition of binding in the presence of 3-O-MG does not support this possibility as 3-O-MG is not a substrate for hexokinase (Sols and Crane, 1954), although the possibility that 3-O-MG was binding to an inactive site of hexokinase was not tested here. The fact that there was no detectable formation of D-[^{3}H]glucose-6-phosphate, measured by ion exchange chromatography and liquid scintillation spectrometry, during these studies was also evidence against hexokinase as a role for the binding site. Two additional studies were done to test whether the specific D-[^{3}H]glucose binding was to hexokinase. These studies were based on the known subcellular distribution of hexokinase: in the cytosol and associated with the mitochondrial membrane (Bachelard, 1967; Rose and Warms, 1967; Wilson, 1968). Several endogenous compounds, including glucose-6-phosphate (G-6-P), ATP, and ADP (Rose and Warms, 1967; Wilson, 1968) influ-

ence this subcellular distribution. Furthermore, G-6-P is a non-competitive inhibitor of hexokinase while ATP is a cosubstrate. The concentration of ATP which caused decreased binding of D-[^{3}H]glucose to synaptic membranes was 1.0 mM, and was comparable to the concentration shown to cause solubilization of hexokinase (Rose and Warms, 1967; Wilson, 1968). The concentrations used here of G-6-P and ATP are similar to the affinity constants for these compounds with hexokinase (Wilson, 1968; Chou and Wilson, 1974). All of these results other than the 3-O-MG data and the lack of metabolism are consistent with the possibility that the binding site is hexokinase.

In summary, I have demonstrated D-[^{3}H]glucose binding to a crude membrane fraction prepared from rat cerebral cortex. Binding was displaceable, saturable, and abolished by heat. The equilibrium constants estimated for binding were K_D = 0.47 μM and B_{max} = 0.22 pmoles per mg protein. The rate of dissociation was sixty times the rate of association. At least two possible roles for this binding site were supported by experimental findings. That the binding site was a carrier molecule for D-glucose was supported by the selectivity and stereospecificity of binding. Alternatively, the possibility that binding was to hexokinase was also supported by the stereospecificity and selectivity, and by the effects of G-6-P, ATP, and ADP in decreasing the specific binding. While these results have been interpreted as evidence for a recognition site for D-glucose in crude membranes derived from rat brain (Gold and Kyle-Lillegard, 1983), elucidation of the role of this site awaits further study.

The glucose carrier protein has been partially purified from other tissue systems (Shanahan and Czech, 1977; Kahlenberg, 1978; Lee and Lip-

mann, 1978), and reconstituted into liposomes and other artificial lipid bilayer preparations (Kasahara and Hinkle, 1976; Crane et al., 1978). Brain hexokinase has also been purified (Needels and Wilson, 1983). Future studies of the binding phenomenon in brain membranes should be done to obtain more evidence in support of a carrier-molecule role for the binding site. Lack of hexokinase activity in the membrane preparation, and inhibition of binding by cytochalasin B would provide evidence for this role. If the binding of D-[^{3}H]glucose to membranes represented binding to a carrier molecule, and this binding site were purified, numerous studies could then be done to learn more about glucose binding and glucose uptake in brain.

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